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A Significant Relationship between Plasma Vitamin C Concentration and Physical Performance among Japanese Elderly Women

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Background. Maintenance of physical performance could improve the quality of life in old age. Recent studies suggested a beneficial relationship between antioxidant vitamin (eg, vitamin C) intake and physical performance in elderly people. The purpose of this study was to examine the relationship between plasma vitamin C concentration and physical performance among Japanese community-dwelling elderly women.

Methods. This is a cross-sectional study involving elderly females residing in an urban area in Tokyo, Japan, in October 2006. We examined anthropometric measurements, physical performance, lifestyles, and plasma vitamin C concentration of participants.

Results. A total of 655 subjects who did not take supplements were analyzed. The mean age (±standard deviation) of participants was 75.7 ± 4.1 years in this study. The geometric mean (geometric standard deviation) of plasma vitamin C concentration was 8.9 (1.5) μg/mL. The plasma vitamin C concentration was positively correlated with handgrip strength, length of time standing on one leg with eyes open and walking speed, and inversely correlated with body mass index. After adjusting for the confounding factors, the quartile plasma vitamin C level was significantly correlated with the subject's handgrip strength (p for trend = .0004) and ability to stand on one leg with eyes open (p for trend = .049).

Conclusions. In community-dwelling elderly women, the concentration of plasma vitamin C related well to their muscle strength and physical performance.

Key Words: Plasma vitamin C—Physical performance—Elderly women—Japanese.

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PHYSICAL performance and physical ability are the most important indicators of health status in elderly people and are also closely related to the quality of life. Declines in physical performance and physical activity, whether from specific disease, fall, fracture, poor nutrition, or aging itself, are associated with future disability, morbidity, and death (1,2).

In recent years, many studies have examined the roles of diet, protein, and vitamins in physical performance and physical activity(3–5). Several studies have associated low serum albumin concentration with deteriorated muscle strength and function (6,7). Some other studies have examined the relationship between serum vitamin D level and physical performance such as muscle mass, muscle strength, handgrip, walking speed, and functional capacity (8,9). Cesari et al. (3) examined the relationship between antioxidant vitamin intake (vitamin C, vitamin E, β-carotene, and retinol) and physical performance in elderly people and showed significant positive correlations between most antioxidants, especially vitamin C, and higher skeletal muscular strength in this group of people.

There are a number of mechanistic hypotheses about the potential beneficial effects of antioxidant vitamins(10–12). Vitamin C, vitamin E, β-carotene, and retinol are important antioxidants that are not synthesized by humans and, therefore, are mainly supplied via dietary intake. Vitamin C

(ascorbic acid) is a water-soluble antioxidant present in the cytosol and extracellular fluid and can directly react with free radicals such as superoxide ($O_2 \cdot^-$) and hydroxyl radicals ($\cdot OH$) (13,14). Each one of these oxygen-derived intermediates is considered highly reactive because of their unstable electron configurations, which could attract electrons from other molecules, resulting in another free radical that is capable of reacting with yet another molecule. This chain reaction is thought to contribute to lipid peroxidation, DNA damage, and protein degradation during oxidative stress. Oxidative damage is thought to play an important role in the age-related decline of functional activity in human skeletal muscle (15). Concentration of plasma vitamin C, which has potent antioxidant activity, is known to increase after exercise (4).

An increase in the amount of blood vitamin C content has been used as an indicator of increased oxidative reaction (11). Previous studies have examined the effects of vitamin C supplementation on physical performance and exercise (4,11). Although findings from some of the previous studies do not support any beneficial effect of increased antioxidant intake on physical performance, other studies have shown improved recovery from exercise with antioxidant intake and have also shown a preventive role of antioxidant supplementation against oxidative damage. These studies were carried out on athletes after heavy exercise. So far, however, there has been no study examining the relationship between physical performance and blood levels of vitamin C, which may be a more direct marker of the antioxidative ability of the human body.

The present study, to the best of our knowledge, is the first report that examines the relationship between plasma vitamin C concentration and physical performance in Japanese community-dwelling elderly women.

Subjects and Methods

Study Subjects

The present cross-sectional study was carried out as part of a project involving mass health examination of community-dwelling people ("Otasha-kenshin" in Japanese) aged 70 years and older living in Itabashi-ku, Tokyo. "Otasha-kenshin," which literally means "health examination for successful aging," is a comprehensive health examination program for community-dwelling older adults aimed at preventing geriatric syndromes including falls and fractures, incontinence, mild cognitive impairment, depression, and undernutrition (16).

The eligible subjects were all female residents, aged between 70 and 84 years, living in the Itabashi area, an urban part of Itabashi-ku, Tokyo, Japan in October 2006. The population of women belonging to this age range and residing in the Itabashi area was 5937, and they were recruited by invitation through postal mail. Of them, 1,112 women applied for admission and 957 women ultimately participated in this study. The participants who were taking vitamin C supplements (n = 238) were excluded from the primary analyses for examination of the relationship between plasma vitamin C and physical performance because intake of supplements could strongly influence the plasma vitamin C level. Thus, data from 655 subjects were ultimately used for the primary analysis. However, data from the 238 supplement users were also used for subanalysis to determine whether any relationship exists between vitamin C supplementation and physical performance.

All participants were examined at the Tokyo Metropolitan Institute of Gerontology's hall. Physical performance, blood examinations, lifestyle assessments, and anthropometric measurements were performed as described below (9).

The present study was approved by the ethics review committee of the Tokyo Metropolitan Institute of Gerontology. All subjects gave written informed consent.

Anthropometric Measurements

Height and weight of each participant were measured, and body mass index was defined as weight/height^2 (kg/m^2). Body composition measurements (percent body fat) were obtained by segmental bioelectrical impedance using eight tactile electrodes according to the manufacturer's instructions (In Body 3.0; Biospace, Seoul, Korea). Measurements for the triceps surae muscles were taken between the knee and the ankle, at the level of maximum circumference of the medial and anterior calf of the left leg of each participant at sitting position.

Physical Performance

Physical performance was assessed by muscle strength (handgrip strength), balance capability, and usual and maximal walking speeds, without prior practice before the actual measurements. These assessments are routinely conducted for the elderly community as described previously (9). Handgrip strength (kg) was measured once for the dominant hand with the subjects in a standing position using a Smedley's Hand Dynamometer (Yagami, Tokyo, Japan). Grip devices were calibrated with known weights. Subjects held the dynamometer at thigh level and were encouraged to exert the strongest possible force. Balance capability was measured in terms of the length of time standing on one leg, that is, we asked the subjects to look straight ahead at a dot 1 m in front of them and to stand on the preferred leg with their eyes open and hands down alongside the trunk. The time until balance was lost (or maximum 60 seconds) was recorded. We used the better of two trials in the analysis. To determine the walking speed, participants were asked to walk on a flat surface at their "usual and maximum walking speeds." Two marks were used to delineate the start and end of a 5-m path. The start mark was preceded by a 3-m approach to ensure that the participants achieved their pace of usual or maximum before entering the test path. The participants were also instructed to continue walking past the end of the 5-m path for a further 3 m to ensure that their walking pace was maintained

throughout the test path. The time taken to complete the 5-m walk was measured by an investigator and used for analysis. Walking test at maximum speed was repeated twice, and the faster speed was recorded for the test.

All physical performance tests were performed between 9 AM and 4 PM during the day. We have no data on the reproducibility of the measurements. To reduce interexaminer variation, each test was conducted by the same staff member specifically trained for this study.

Blood Examinations

Blood samples (nonfasting) were collected from the subjects between 9 am and 4 pm during the day. There was no difference in mean plasma vitamin C concentration with regard to the time of collection (data not shown). Venous blood samples were drawn into Ethylene diamine tetraacetic acid tubes. Plasma was then obtained by centrifugation at 3,000 rpm for 15 min at 4°C and subsequently used for biochemical assays. Plasma was treated with Ethylene diamine tetraacetic acid to prevent the spontaneous vitamin C degradation. Next, 100 μl of the plasma was dispensed into storage tubes, to which 450 μl of 3% metaphosphoric acid solution was added, and the mixture was stored at −80°C until further use. Vitamin C concentration was determined by an High performance liquid chromatography-electrochemical detection–based method (17). The analysis was carried out centrally in our laboratory. Serum albumin concentration was measured by the Bromocresol Green method (Special Reference Laboratories Inc., Tokyo, Japan). The coefficient of variation for serum albumin found using this method was less than 1% (9).

Lifestyle Assessment

Information regarding the participants' general health (such as medical history, smoking habits, alcohol drinking habits, regular exercise habits, vegetable intake, fruit intake and use of vitamin C supplement) was collected by interview, and history of medical conditions including hypertension, stroke, heart attack, diabetes mellitus, and hyperlipidemia was self-reported.

Alcohol drinking habits of the subjects were classified as nondrinker, current drinker, or ex-drinker. Smoking habits of the subjects were classified using three categories: never smokers, current smokers, and ex-smokers. The frequency of vegetable and fruit intake was asked using four categories: almost every day, once every two days, once or twice per week, and almost never. Subsequently, for analysis, the categories were summarized as almost every day and others.

Statistical Analysis

Data were summarized as mean and standard deviation or percentage values. The data of plasma vitamin C concentration was logarithmically transformed to approximate a normal distribution and was summarized as the geometric mean and geometric standard deviation.

Table 1. Characteristics of Study Subjects ($N = 655$)

Characteristic	Mean (*SD*)
Age (y)	75.7 (4.1)
Height (cm)	149.1 (5.7)
Weight (kg)	51.0 (8.3)
Body mass index (kg/m^2)	22.9 (3.4)
Triceps surae muscle (cm)	33.1 (2.8)
Plasma vitamin C (μg/ml)*	8.9 (1.5)
Serum albumin (mg/dL)	4.3 (0.2)
Body composition	
Percent body fat (%)	32.2 (7.0)
Physical performance tests	
Handgrip strength (kg)	18.7 (4.4)
One leg standing with eyes open (s)	35.2 (23.5)
Usual walking speed (m/s)	1.2 (0.3)
Maximal walking speed (m/s)	1.8 (0.4)
	%
Medical history	
Hypertension	50.7
Stroke	6.6
Heart attack	21.2
Diabetes mellitus	9.0
Hyperlipidemia	34.7
Alcohol drinking habit	
Current	25.3
Former	5.0
Never	69.6
Smoking habit	
Current	3.7
Former	5.7
Never	90.7
Regular exercise habit	
Yes	69.2
No	30.8
Vegetable intake	
Everyday	84.2
Others†	15.8
Fruit intake	
Everyday	81.8
Others†	18.2

Notes: Data of vitamin C supplement users were excluded.
*The geometric mean and geometric *SD*.
†Including participants taking vegetables/fruits not everyday or almost never.

The age-adjusted Pearson's correlation coefficient between the plasma vitamin C concentration and other factors were calculated. The least square means and SEs adjusted for potential confounders were calculated and compared between categories by analysis of covariance. To examine the relationship between plasma vitamin C concentration and physical performance, statistical adjustment was done by analysis of covariance for variables (except for other physical performance variables) that were correlated to plasma vitamin C concentration with $p < .20$. The same analyses were repeated for the 238 users of vitamin C supplement. All statistical analyses were performed using the SAS (version 9.0; SAS Institute Inc., NC).

Results

Table 1 summarizes the basic characteristics of the subjects. As shown, the mean age (±standard deviation) of the

Table 2. Correlation between Plasma Vitamin C Concentration and Selected Factors ($N = 655$)

Factor	Correlation*	
	r	p
Age	−0.004	.91
Height	0.04	.27
Weight	−0.05	.19
Body mass index	−0.08	.054
Triceps surae muscle	0.001	.98
Serum albumin	−0.04	.33
Percent body fat	−0.12	.002
Handgrip strength	0.16	<.001
One leg standing with eyes open	0.15	<.001
Usual walking speed	0.14	<.001
Maximal walking speed	0.09	.036

Notes: Number of subjects is slightly different for the selected factors because of missing values.

*Age-adjusted Pearson's correlation coefficient between logarithm of vitamin C concentration and each factor.

subjects was 75.7 ± 4.1 years. The geometric mean (geometric standard deviation) of plasma vitamin C concentration was 8.9 (1.5) μg/mL. The prevalence of women eating vegetables everyday was 84.2% and those eating fruits everyday was 81.8%.

The age-adjusted geometric mean of plasma vitamin C concentration was significantly lower in subjects who had a medical history of hypertension (8.53 vs 9.22, $p = .0015$) and diabetes mellitus (7.59 vs 9.00, $p = .002$) as compared with those who did not. A history of stroke, heart attack, or hyperlipidemia was not associated with plasma vitamin C concentration. Subjects who took fruits every day had a significantly higher concentration of vitamin C than those who did not (9.14 vs 7.78, $p < .0001$). Vegetable intake, alcohol drinking habit and smoking habit were not related to plasma vitamin C concentration (not shown in table).

Table 2 shows the age-adjusted correlations between the plasma vitamin C concentration and selected factors. As shown, the plasma vitamin C concentration was positively but modestly correlated with handgrip strength, length of time standing on one leg with eyes open, as well as usual walking speed and maximal walking speed, and modestly inversely correlated with body mass index and percent body fat of the subjects.

Table 3 shows the relationship between plasma vitamin C concentration and each physical performance after adjusting for confounding factors. Results obtained after the adjustment for potential confounders confirmed that the plasma vitamin C concentration was correlated with the handgrip strength independently from the other factors (eg, p for trend = .0004 after adjusting for age, body mass index, percent body fat, hypertension, diabetes mellitus, and fruit intake; Table 3). There was also a significant relationship between the plasma vitamin C level and the subject's length of time standing on one leg with eyes open after adjustments for age, body mass index, percent body fat, hypertension, diabetes mellitus, and fruit intake (Table 3; p for trend = .049). We did not observe any significant association between the plasma vitamin C level and the usual or the maximal walking speed of the subjects.

A subanalysis using data from the 238 vitamin C supplement users showed almost null relationship between handgrip strength and plasma vitamin C concentration (data not shown).

Discussion

A previous study has shown an association between higher daily dietary intake of vitamin C and skeletal muscle strength in elderly people (3). Results described in the present study indicated that plasma vitamin C concentration was positively related with muscle and physical performance in community-dwelling elderly women. To the best of our knowledge, this is the first study showing a significant

Table 3. Relationship between Plasma Vitamin C Concentration and Physical Performance Adjusted for Potential Confounder

Physical performance	Quartile of plasma vitamin C level				
	Q1	Q2	Q3	Q4	
	Mean ± *SE*	Mean ± *SE*	Mean ± *SE*	Mean ± *SE*	p for trend
Handgrip strength (kg), *N*	154	159	154	152	
Age adjusted	17.70 ± 0.34	18.75 ± 0.33	18.75 ± 0.34	19.60 ± 0.34	.0001
Multivariate adjusted*	17.83 ± 0.34	18.83 ± 0.32	18.89 ± 0.33	19.60 ± 0.33	.0004
One leg standing with eyes open† (s), *N*	162	163	164	161	
Age adjusted	31.44 ± 1.71	33.98 ± 1.70	37.70 ± 1.70	37.83 ± 1.71	.003
Multivariate adjusted*	33.39 ± 1.74	34.08 ± 1.67	37.63 ± 1.67	37.50 ± 1.70	.049
Usual walking speed (m/s), *N*	146	154	145	147	
Age adjusted	1.13 ± 0.02	1.19 ± 0.02	1.23 ± 0.02	1.21 ± 0.02	.008
Multivariate adjusted*	1.18 ± 0.02	1.19 ± 0.02	1.22 ± 0.02	1.21 ± 0.02	.23
Maximal walking speed (m/s), *N*	146	154	154	147	
Age adjusted	1.70 ± 0.03	1.76 ± 0.03	1.82 ± 0.03	1.76 ± 0.03	.15
Multivariate adjusted*	1.76 ± 0.03	1.77 ± 0.03	1.80 ± 0.03	1.75 ± 0.03	.94

Notes: Values are least squares mean and *SE* adjusted for the factors by analysis of covariance. Q1–Q4: first to fourth quartile groups of plasma vitamin C concentration, respectively.

*Adjusted for age, body mass index, percent body fat, hypertension, diabetes mellitus and **fruit intake**.

†Length of time standing on one leg with eyes open.

correlation between plasma vitamin C concentration and handgrip strength and ability to stand on one leg with eyes open. We, however, were unable to find any relationship between skeletal muscle mass and plasma vitamin C concentration. Handgrip strength has been found to correlate well with the strength of other muscle groups and is thus a good indicator of overall strength (18). Consistent with this idea, handgrip strength was found to be a strong and consistent predictor of all-cause mortality and morbidity of Activities of Daily Living in middle-aged people (19). The handgrip test is considered an easy and inexpensive screening tool to identify elderly people at risk of disability. Handgrip strength, an indicator of overall muscle strength, is thought to predict mortality through mechanisms other than underlying disease that could cause muscle impairment (18,19). The one leg standing test is one of the balance tests (20). The test is a clinical tool to assess postural steadiness in a static position by quantitative measurement. Many studies have shown that the decreased one leg standing time is associated with declines in Activities of Daily Living and increases in other morbidities including osteoporosis and fall (20).

Our findings suggest that vitamin C may play an important role in maintaining physical performance and thereby may help to improve healthy life expectancy in the elderly. However, the usual and maximal walking speeds did not relate to plasma vitamin C concentration. Walking speed test may be an efficient tool in screening older persons with higher risk of mortality and may easily identify high-risk groups in the community (21). Walking is a rhythmic, dynamic, and aerobic activity of the large skeletal muscles that confers multifarious benefits with minimal adverse effects. Muscles of the legs, limbs, and lower trunk are strengthened, and the flexibility of their joints are preserved (22). One of the reasons why walking speed was not related to vitamin C concentration may be because walking requires coordinated movements of arms, legs, and many parts of the body rather than a simple muscle and balance function. Previous reports showed that walking balance function did not correlate with standing balance function (23). Although we did not find any clear association between walking and plasma vitamin C concentration in this study, vitamin C may still have effects on relatively simple strength and balance functions.

One of the possible explanations for the observed relationship between vitamin C and physical performance, especially handgrip strength and the ability to stand on one leg with eyes open, may be the potential protective effects of the antioxidant vitamins against muscle damage (4,11). Vitamin C is a six-carbon lactone that is synthesized from glucose in the liver of most mammalian species, but not in humans (12). Vitamin C is an antioxidant because, by donating its electrons, it prevents other compounds from being oxidized (12). Thus, vitamin C readily scavenges reactive oxygen and nitrogen species, thereby effectively protects other substrates from oxidative damage (10,24). Although habitual exercise reduces systemic inflammation and oxidative stress as the production of endogenous antioxidants are enhanced, acute exercise increases the generation of oxygen-free radicals and lipid peroxidation (4,25). Strenuous physical performance can increase oxygen consumption by 10- to 15-folds over the resting state to meet the energy demands and results in muscle injury (26). Prolonged submaximal exercise was shown to increase the amount of both whole-body and skeletal muscle lipid peroxidation by-products; in the case of the former, the increase was indicated by greater exhalation of pentane but not of ethane (4,27,28). Supplementation with vitamin C was shown to decrease the exercise-induced increase in the rate of lipid peroxidation (27,28). Several studies suggested that oxidative damage may play a crucial role in the decline of functional activity in human skeletal muscle with normal aging (15). Consistent with this idea, several studies showed significantly lower plasma vitamin C level in the elderly population than in the younger adult population (29–31). Because the plasma vitamin C levels in these apparently healthy elderly persons rose markedly after an oral dose of vitamin C, their initially low plasma levels can be attributed to the low intake rather than to an age-related physiological defect.

In fact, the relationship between handgrip strength and plasma vitamin C concentration was significantly different between supplement users and nonusers, that is, an almost null relationship in the former and a positive relationship in the latter (data not shown). This finding suggested that vitamin C supplementation did not have any beneficial effect on the physical performance and muscle strength despite the increased plasma level of vitamin C. A number of studies reported that vitamin C supplement users had significantly higher blood vitamin C concentration than non-users (29, 32, 33). Several studies have examined the effects of exercise on changes in the serum vitamin C concentration (34–36). Some other experimental studies have shown that vitamin C supplementation can reduce symptoms or indicators of exercise-induced oxidative stress (37–40). However, the results regarding vitamin C supplementation are equivocal, and most well-controlled intervention studies report no beneficial effect of vitamin C supplementation on either endurance or strength performance (41,42). Likewise, vitamin C restriction studies showed that a marginal vitamin C deficiency did not affect the physical performance (43). Although evidence from a number of studies show that vitamin C is a powerful antioxidant in biological systems in vitro, its antioxidant role in humans has not been supported by currently available clinical studies.

Vitamin C is especially plentiful in fresh fruits and vegetables. Plasma vitamin C concentration may be merely a marker for intake of other nutrients that are abundant in fruits and vegetables. However, the statistical adjustment for fruit intake did not attenuate the relationship between plasma vitamin C and physical performance (Table 3), suggesting that vitamin C did have some beneficial effects

independently of other nutrients. A number of biochemical, clinical, and observational epidemiologic studies have indicated that diets rich in fruits, vegetables, and vitamin C may be of benefit for the prevention of chronic diseases such as cardiovascular disease and cancer (44,45). Several cohort studies have examined associations between plasma vitamin C concentration and mortality from stroke or coronary heart disease (30,46,47). The effects of vitamin C supplementation are, however, still unclear. A pooled study suggested reduced incidences of coronary heart disease events with higher intake of vitamin C supplement (48), while another study showed that a high intake of vitamin C supplement is associated with an increased risk of mortality due to cardiovascular diseases in postmenopausal women with diabetes (49). A randomized placebo controlled 5-year trial, however, did not show any significant reduction in the mortality from, or incidence of, any type of vascular disease or cancer (50). These studies, in fact, have failed to demonstrate any benefit from such supplementation.

There are a number of potential weaknesses in our study that should be mentioned here. The subjects used in this study were not selected randomly from the study population, and they may be relatively healthy elderly women who were able to come to the health examination hall from their homes. A previous study assessed the correlation of antioxidants with physical performance and muscular strength (3) and demonstrated that a higher daily intake of vitamin C and carotene associated with skeletal muscle strength. However, we have no data regarding the presence of other dietary antioxidants in blood such as vitamin E, retinol, and carotene. In our questionnaire, participants were asked to respond "Yes" or "No" to whether they took supplements, and not about the frequency and quantity of intake of the supplements. Thus, we were unable to examine the reason why plasma vitamin C was not related to the handgrip strength in the supplement users by considering the dose of vitamin C they took.

This study was a cross-sectional study and, therefore, does not provide cause/effect relationships, although we demonstrated a significant correlation between physical performance and concentration of plasma vitamin C. Therefore, longitudinal follow-up studies and controlled clinical trials are necessary to confirm the role of plasma vitamin C and physical performance of the elderly women. These limitations should be considered in future studies.

In conclusion, we found a strong correlation of a higher plasma vitamin C concentration with handgrip strength and one leg standing time in community-dwelling elderly women. Although the elderly are prone to vitamin C deficiency, and they appear to have a higher dietary requirement for vitamin C, the beneficial effects of vitamin C supplementation to maintain physical performance in elderly people are equivocal and thus, need further in-depth studies.

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Senescence Marker Protein-30/Gluconolactonase Deletion Worsens Glucose Tolerance through Impairment of Acute Insulin Secretion

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Senescence marker protein-30 (SMP30) is an androgen-independent factor that decreases with age. We recently identified SMP30 as the lactone-hydrolyzing enzyme gluconolactonase (GNL), which is involved in vitamin C biosynthesis in animal species. To examine whether the age-related decrease in SMP30/GNL has effects on glucose homeostasis, we used SMP30/GNL knockout (KO) mice treated with L-ascorbic acid. In an ip glucose tolerance test at 15 wk of age, blood glucose levels in SMP30/GNL KO mice were significantly increased by 25% at 30 min after glucose administration compared with wild-type (WT) mice. Insulin levels in SMP30/GNL KO mice were significantly decreased by 37% at 30 min after glucose compared with WT mice. Interestingly, an insulin tolerance test showed a greater glucose-lowering effect in SMP30/GNL KO mice. High-fat diet feeding severely worsened glucose tolerance in both WT and SMP30/GNL KO mice. Morphometric analysis revealed no differences in the degree of high-fat diet-induced compensatory increase in β-cell mass and proliferation. In the static incubation study of islets, insulin secretion in response to 20 mM glucose or KCl was significantly decreased in SMP30/GNL KO mice. On the other hand, islet ATP content at 20 mM in SMP30/GNL KO mice was similar to that in WT mice. Collectively, these data indicate that impairment of the early phase of insulin secretion due to dysfunction of the distal portion of the secretion pathway underlies glucose intolerance in SMP30/GNL KO mice. Decreased SMP30/GNL may contribute to the worsening of glucose tolerance that occurs in normal aging. (*Endocrinology* 151: 0000–0000, 2010)

Senescence marker protein-30 (SMP30), a 34-kDa protein originally identified in rat liver, is a novel molecule whose expression decreases with age in a sex-independent manner (1, 2). SMP30 transcripts have been detected in multiple tissues, and its amino acid alignment reveals a highly conserved structure among humans, rats, and mice (2). We previously reported that SMP30 participates in Ca^{2+} efflux by activating the calmodulin-dependent Ca^{2+} pump in HepG2 cells and renal tubular cells, conferring on these cells a resistance to injury caused by high intracellular Ca^{2+} concentrations (3, 4). Recently, we identified SMP30 as glucolactonase (GNL), which is involved in L-ascorbic acid biosynthesis in mammals, although human beings are unable to synthesize vitamin C *in vivo* because there are many mutations in their gulonolactone oxidase gene, which catalyzes the conversion of L-gulono-γ-lactone to L-ascorbic acid (5).

To clarify the causal relationship between decreased SMP30/GNL and age-associated physiological changes, we created SMP30/GNL knockout (KO) mice (6). The

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Abbreviations: AUC, Area under the curve; BrdU, 5-bromo-2-deoxyuridine; GNL, gluconolactonase; HFD, high-fat diet; KO, knockout; NEFA, nonesterified fatty acid; SD, standard diet; SMP30, senescence marker protein-30; WT, wild-type.

livers of SMP30/GNL KO mice were highly susceptible to TNFα- and Fas-mediated apoptosis (6). In addition, their livers showed abnormal accumulation of triglycerides, cholesterol, and phospholipids (7). The lungs of SMP30/GNL KO mice developed alveolar air sac enlargement similar to the senile lung syndrome seen in some elderly people (8, 9). The deposition of lipofuscin, an aging marker, was observed in renal tubular epithelial cells in these mice (2). Furthermore, SMP30/GNL in the brain and lungs has been proposed to have protective effects against oxidative stress associated with aging (9, 10). Although the physiological function of SMP30/GNL is still not entirely clear, our studies using SMP30/GNL KO mice have revealed that a reduction in SMP30/GNL expression may account for the age-associated deterioration of cellular function and the enhanced susceptibility to harmful stimuli in aged tissue. Also, these mice displayed symptoms of scurvy when fed a vitamin C-deficient diet (5, 11–13).

The reduction of carbohydrate metabolism in the elderly is one of the hallmarks of the aging process, and substantial evidence shows that increasing age is associated with worsened glucose tolerance and type 2 diabetes (14, 15). However, the molecular abnormalities that occur in the elderly have not been fully elucidated. Because SMP30/GNL KO mice show phenotypic changes that mimic the premature aging process, we hypothesized that a reduction in SMP30/GNL expression could be linked to the worsening of glucose tolerance that occurs with normal aging. The purpose of this study was to examine the role of SMP30/GNL in glucose homeostasis using SMP30/GNL KO mice.

Materials and Methods

Animals

SMP30/GNL KO mice were generated as described earlier by gene targeting in the background strain C57BL/6 (6). All studies were performed on male mice using age-matched, wild-type (WT) male C57BL/6CrSlc mice (Shimizu Laboratory Supplies Co., Ltd., Kyoto, Japan) as controls. Mice were fed a high-fat diet (HFD 32; 507.6 kcal/100 g, fat kcal 56.7%; CLEA Japan, Tokyo, Japan) or a standard diet (SD; 346.8 kcal/100 g, fat kcal 10%, CLEA Japan) for 8 wk from 7 wk of age. As we have previously reported, SMP30/GNL KO mice cannot synthesize vitamin C *in vivo* because SMP30/GNL is a key enzyme involved in vitamin C biosynthesis (5). To avoid the effects of vitamin C deficiency, L-(+)-ascorbic acid (vitamin C, 1.5 g/liter) and 10 μM EDTA were added to the drinking water. The water was changed every 3 d until the experiment ended. Mice had free access to water and food and were maintained on a 12-h light, 12-h dark cycle with a controlled temperature. All experimental procedures were approved by the Committee for Animal Research, Kyoto Prefectural University of Medicine.

Analytic procedures and glucose and insulin tolerance tests

Blood glucose levels were measured using a glucometer (Gultest Ace; Sanwa Kagaku Kenkyusho Co., Ltd., Nagoya, Japan). To measure plasma lipids, blood was collected by cardiac puncture after an overnight fast just before the tissue collection. Plasma nonesterified fatty acid (NEFA), triglycerides, and total cholesterol were measured by the enzymatic method using an autoanalyzer. Intraperitoneal glucose (2 g/kg body weight) and insulin (0.75 U/kg body weight) tolerance tests were performed after 16- and 7-h fasts, respectively, and blood glucose was measured at the time indicated. For insulin release during glucose tolerance testing, the plasma component of blood collected at the 0- and 30-min time points was measured with an insulin enzyme immunoassay system, the Morinaga ultrasensitive mouse insulin assay kit (Morinaga Institute of Biological Science, Inc., Kanagawa, Japan).

Tissue collection and histological assessment of pancreatic islets

After an overnight fast, mice were killed by administration of an overdose of sodium aminobarbital. After blood collection by cardiac puncture, the lateral epididymal and inguinal sc adipose tissue depots were removed and weighed. A portion of the liver was used for measurement of total vitamin C content.

The whole pancreas was removed, cleared of fat and lymph nodes, weighed, fixed in 10% formalin solution, and embedded in paraffin. Pancreatic sections were prepared and stained with hematoxylin and eosin or an antibody against insulin using the Histofine mouse stain kit with a mouse antihuman insulin antibody (Nichirei Biosciences Inc., Tokyo, Japan). For morphometric analysis of β-cell mass, pancreatic sections 250 μm apart taken from three different levels of the pancreatic tissue block (five pancreases per experimental group) were examined. Nonoverlapping images were captured with a digital camera (Sony DXC-S500/OL; Sony, Tokyo, Japan). The β-cell area and the section area were analyzed using NIH Image J version 1.36b software. The β-cell mass was calculated by multiplying the pancreas weight by the percentage of β-cell area per pancreas.

To evaluate cell replication per islet, we performed labeling of proliferating cells with the DNA precursor analog 5-bromo-2-deoxyuridine (BrdU). Mice were injected ip with BrdU (1 mg/mouse; BD Biosciences, San Diego, CA) 12 h before being killed. After tissue processing as described above, immunostaining for BrdU was performed with a BrdU *in situ* detection kit (BD Biosciences) (16). All visible islets for each pancreatic section (three sections per animal) were analyzed to determine the total number of BrdU-positive cells and total islet count per section.

Measurement of pancreatic insulin content

Pancreatic insulin content was determined as previously described (17). Briefly, a portion of the pancreatic tail was homogenized in acidic ethanol (0.18 mol/liter HCl in 95% ethanol) and was extracted for 24 h at 4 C. The homogenate was centrifuged at 2000 × *g* for 15 min. Insulin levels in the supernatant were assayed as described above.

Measurement of total vitamin C levels in the liver

Livers were homogenized in 14 vol 5.4% metaphosphate, and then the homogenate was centrifuged at 21,000 × *g* for 15 min

at 4 C. Ascorbic acid in samples was treated with 0.1% dithiothreitol to reduce the dehydroascorbic acid to ascorbic acid and was analyzed by HPLC using an Atlantis dC18 5-μm column (4.6 × 150 mm; Nihon Waters, Tokyo, Japan) (11). The mobile phase was 50 mM phosphate buffer (pH 2.8), 0.2 g/liter EDTA, and 2% methanol at a flow rate of 1.3 ml/min, and electrical signals were recorded by using an electrochemical detector with a glassy carbon electrode at +0.6 V.

Static insulin secretion from isolated islets

Male SD-fed SMP30/GNL KO mice and C57BL/6CrSlc mice (14–15 wk of age) were used for islets isolation. Islets were isolated using collagenase (type V collagenase; Sigma Chemical Co., St. Louis, MO), digested in Hanks' buffer, followed by separation of islets from exocrine tissue in a Histopaque (Histopaque 1077; Sigma) gradient (18). Islets of similar size were hand picked under a stereomicroscope into groups (n = 5) of five islets in triplicate. The islets were preincubated for 60 min at 37 C in Krebs-Ringer bicarbonate HEPES buffer (equilibrated with 95% O_2 and 5% CO_2, pH 7.4) supplemented with 2 mg/ml BSA (fraction V; Sigma) and 2 mM glucose. After preincubation, five islets were incubated with 200 μl of the same buffer for 15 min. After samples from the buffer were removed for measurement of insulin, the islets were incubated in the presence of 20 mM glucose or 20 mM KCl plus 2 mM glucose for another 15 min. At the end of this period, the supernatant was collected. All samples were stored at −80 C until the insulin assay (Morinaga ultrasensitive mouse insulin assay kit).

ATP measurement from isolated islets

Cultured islets were preincubated at 37 C for 60 min in Krebs-Ringer bicarbonate HEPES buffer with 2 mM glucose, and then triplicate batches of 10 islets were incubated at 2 or 20 mM glucose for another 60 min. ATP was extracted from islets according to the methods described by Uchizono *et al.* (19). ATP levels were measured using the Enliten ATP assay system (Promega, Madison, WI) with a bioluminometer (GloMax 20/20n luminometer; Promega).

Statistical analysis

The data are expressed as means ± SE. Significance was determined by one-way ANOVA with Dunn's multiple comparisons *post hoc* or unpaired Student's *t* test where appropriate. A two-way ANOVA was used to compare the glucose and insulin levels on the same time point in ip glucose tolerance test. A value of $P < 0.05$ was considered to be significant.

Results

Energy intake, body weight, and adipose tissue

The mean energy intakes of the WT mice fed a SD, WT mice fed a HFD, SMP30/GNL KO mice fed an SD, and SMP30/GNL KO mice fed an HFD throughout the study were 9.1 ± 0.1, 13.1 ± 0.2, 8.5 ± 0.1, and 11.8 ± 0.2 kcal/d, respectively. All four groups of mice gained weight; however, the increase in body weight in the HFD-fed WT and HFD-fed SMP30/GNL KO mice was significantly higher than that in SD-fed WT and SD-fed SMP30/GNL KO mice (Fig. 1). The weight gain in the SD-fed WT, SD-fed SMP30/GNL KO, HFD-fed WT, and HFD-fed SMP30/GNL KO mice after 8 wk on the diets was 8.4 ± 0.4, 8.2 ± 0.4, 16.5 ± 0.7, and 14.7 ± 0.7 g/mouse, respectively. The HFD-fed WT mice gained 51% more weight than the SD-fed WT mice, and HFD-fed SMP30/GNL KO mice gained 56% more weight than the SD-fed SMP30/GNL KO mice.

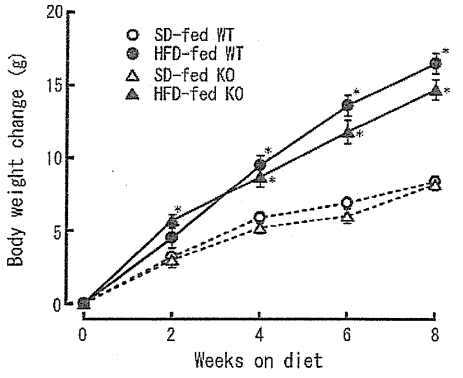

FIG. 1. Body weight changes of SD-fed and HFD-fed WT and SMP30/GNL KO mice. Experimental diets were started at 7 wk of age and continued for 8 wk (until 15 wk of age). *, $P < 0.01$ *vs.* SD-fed WT and SMP30/GNL KO mice. Data are means ± SE (n = 10 per group).

Epididymal fat pads from HFD-fed WT and HFD-fed SMP30/GNL KO mice after 8 wk on the diet were significantly heavier than fat pads from the SD-fed WT and SD-fed SMP30/GNL KO mice (Table 1). The ratio of epididymal fat mass to sc fat mass in HFD-fed WT and HFD-fed SMP30/GNL KO mice was also larger than in SD-fed WT and SD-fed SMP30/GNL KO mice, although the differences were not significant.

Total vitamin C levels in the liver

To examine the vitamin C status of the SMP30/GNL KO mice, we determined the total vitamin C content in the liver after 8 wk on the diets. The total vitamin C levels in the liver from SD-fed and HFD-fed WT mice and SD-fed and HFD-fed SMP30/GNL KO mice were 188.2 ± 12.3, 176.6 ± 8.9, 160.2 ± 7.4, and 150.4 ± 6.9 μg/g tissue, respectively. There were no significant differences among the four groups.

Blood glucose and plasma lipid levels

Fasting glucose levels of HFD-fed WT and HFD-fed SMP30/GNL KO mice after 8 wk on HFD were significantly increased in both groups compared with SD-fed WT and SD-fed SMP30/GNL KO mice (Table 1).

After 8 wk on HFD, HFD-fed WT and HFD-fed SMP30/GNL KO mice showed significant increases in total plasma cholesterol levels from 61–67% compared with SD-fed WT and SD-fed SMP30/GNL KO mice; however, there was no significant difference in plasma total

TABLE 1. Fasting glucose, plasma lipids, and adipose tissue weight after 8 wk on diet (15 wk of age)

	SD		HFD	
	WT	SMP30/GNL KO	WT	SMP30/GNL KO
Fasting glucose (mg/dl)	109.5 ± 3.7	101.6 ± 5.0	129.6 ± 6.4[a]	138.3 ± 6.7[a]
NEFA (mEq/liter)	1487.0 ± 79.0	1439.2 ± 56.6	927.9 ± 40.6[a]	882.1 ± 39.8[a]
Triglycerides (mg/dl)	106.4 ± 3.2	93.8 ± 4.5	74.6 ± 5.4[a]	69.8 ± 4.8[a]
Total cholesterol (mg/dl)	117.3 ± 2.7	108.1 ± 2.4	191.5 ± 10.2[b]	161.5 ± 6.6[a]
Epididymal fat (mg/g BW)	12.6 ± 1.5	9.0 ± 1.0	23.5 ± 1.9[a]	18.8 ± 2.1[a]
sc fat (mg/g BW)	5.9 ± 1.1	3.3 ± 0.5[b]	7.5 ± 0.5	5.5 ± 0.4
Epi/Sub	2.5 ± 0.4	2.9 ± 0.3	3.3 ± 0.4	3.4 ± 0.3

Data are means ± SE from 10 mice. BW, Body weight; Epi/Sub, ratio of epididymal fat mass to sc fat mass.

[a] $P < 0.05$ *vs.* SD-fed WT and SMP30/GNL KO mice.

[b] $P < 0.05$ *vs.* the other three groups.

cholesterol levels between SD-fed WT and SD-fed SMP30/GNL KO mice (Table 1). Compared with the mice fed SD, mice fed HFD had lower triglyceride and NEFA levels. However, there was no difference in triglyceride and NEFA levels between WT and SMP30/GNL KO mice. This paradoxical decrease in triglycerides and NEFAs is consistent with a previous report showing that the C57BL/6 strain has a unique metabolic response to HFD (20). These data indicate that deficiency of SMP30 has no influence on lipid profile either in SD-fed or in HFD-fed animal.

Intraperitoneal glucose tolerance test

Blood glucose levels at 30 min after glucose administration were 25% higher in SD-fed SMP30/GNL KO mice than in SD-fed WT mice ($P < 0.05$, Fig. 2A). And blood glucose levels at 30, 60, and 120 min after glucose administration were significantly higher in HFD-fed WT and HFD-fed SMP30/GNL KO mice than in SD-fed WT and SD-fed SMP30/GNL KO mice. Moreover, the blood glucose levels of HFD-fed SMP30/GNL KO mice at 60 and 120 min after glucose administration were significantly higher than those of HFD-fed WT mice. The areas under the curve (AUC, 0–120 min) in SD-fed and HFD-fed WT mice and SD-fed and HFD-fed SMP30/GNL KO mice were 417.1 ± 18.3, 721.2 ± 41.7, 496.1 ± 36.2, and 900.2 ± 40.6 mg · h/dl, respectively (ANOVA, $P < 0.0001$). It is noteworthy that the AUC did not differ between SD-fed WT and SD-fed SMP30/GNL KO mice ($P = 0.128$). This result indicates that the significantly high blood glucose levels appeared restrictively at 30 min after glucose administration in SD-fed SMP30/GNL KO mice.

There were no significant differences in fasting insulin levels between SD-fed WT and SD-fed SMP30/GNL KO mice (Fig. 2B). However, insulin levels of SD-fed SMP30/GNL KO mice at 30 min after glucose were 37% lower than those of SD-fed WT mice ($P < 0.05$). HFD feeding increased fasting insulin levels in both WT and SMP30/GNL KO mice; however, those of SMP30/GNL KO mice were 39% lower than those of WT mice. Similarly, insulin levels at 30 min after glucose were 27% lower in HFD-fed SMP30/GNL KO mice compared with those of HFD-fed WT mice. The AUC (0–30 min) in SD-fed and HFD-fed WT mice and SD-fed and HFD-fed SMP30/GNL KO mice were 1.09 ± 0.08, 1.86 ± 0.19, 0.71 ± 0.11, and 1.19 ± 0.12 ng · 30 min/ml, respectively (ANOVA, $P < 0.0001$). The AUC in SD-fed SMP30/GNL KO mice was lower than that in SD-fed WT mice, although the difference was not statistically significant ($P = 0.052$).

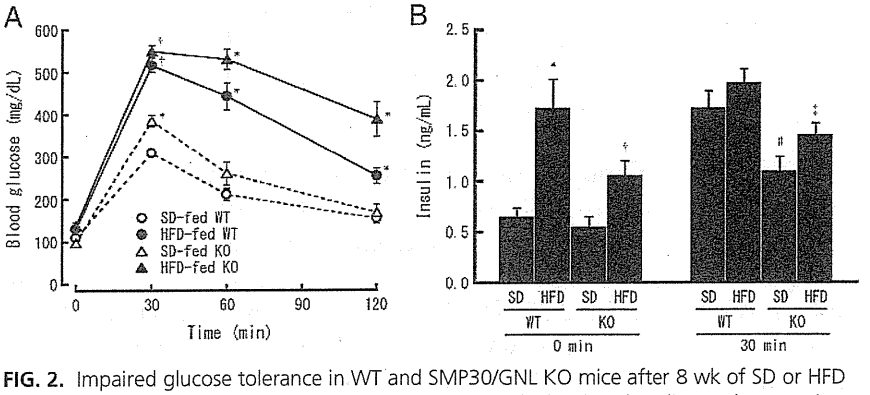

FIG. 2. Impaired glucose tolerance in WT and SMP30/GNL KO mice after 8 wk of SD or HFD feeding. A, Intraperitoneal glucose tolerance test; B, insulin levels at baseline and at 30 min after glucose. *, $P < 0.05$ *vs.* the other three groups; †, $P < 0.05$ *vs.* SD-fed WT and SMP30/GNL KO mice; #, $P < 0.05$ *vs.* SD-fed and HFD-fed WT mice; ‡, $P < 0.05$ *vs.* HFD-fed WT mice. Data are means ± SE (n = 7 per group).

Insulin tolerance test

We next assessed insulin sensitivity using an insulin tolerance test. Blood glucose levels in SD-fed SMP30/GNL KO mice were significantly reduced to 49 and 51% of the levels of SD-fed WT mice after 30 and 60 min, respectively, indicating high peripheral insulin sensitivity (Fig. 3). HFD-fed SMP30/GNL KO mice showed similar insulin tolerance to that of SD-fed WT mice.

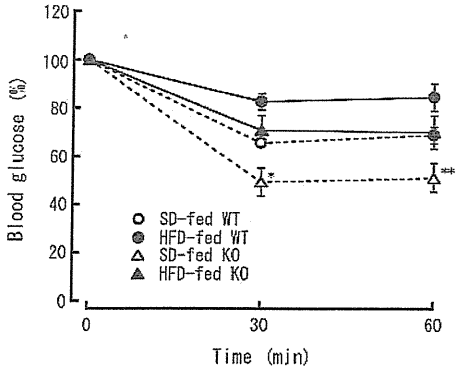

FIG. 3. Intraperitoneal insulin tolerance test after 8 wk of SD or HFD feeding. Data are expressed as percentage of basal (0 min) glucose levels. *, $P < 0.05$ *vs.* HFD-fed WT and SMP30/GNL KO mice; **, $P < 0.05$ *vs.* the other three groups. Data are means ± SE (n = 7 per group).

β-Cell mass and proliferation

The pancreas weight per body weight was significantly lower in SD-fed SMP30/GNL KO mice than in SD-fed WT mice (Table 2). Eight weeks of HFD feeding increased the pancreas weight of SMP30/GNL KO mice to the same level as SD-fed and HFD-fed WT mice. Morphometric analyses of β-cell mass, as assessed by immunohistochemical analysis, revealed no differences between SMP30/GNL KO mice and WT mice (Table 2). HFD feeding significantly increased β-cell mass in both WT and SMP30/GNL KO mice to similar levels. Similarly, there were no significant differences in pancreatic insulin content in response to HFD feeding between WT and SMP30/GNL KO mice (Table 2).

Hematoxylin and eosin staining revealed no differences in islet morphology among the four groups. The proliferation of islet cells, which was determined by the frequency of BrdU-positive cells per islet, in both HFD-fed WT and SMP30/GNL KO mice was significantly higher than in animals fed SD (Fig. 4 and Table 2). In addition, there was no difference in islet proliferation between SMP30/GNL KO and WT mice. This finding demonstrates that the proliferation of β-cells to compensate for increased insulin demand is not impaired in SMP30/GNL KO mice. However, insulin levels of SD-fed SMP30/GNL KO mice at 30 min after glucose were 37% lower than those of SD-fed WT mice.

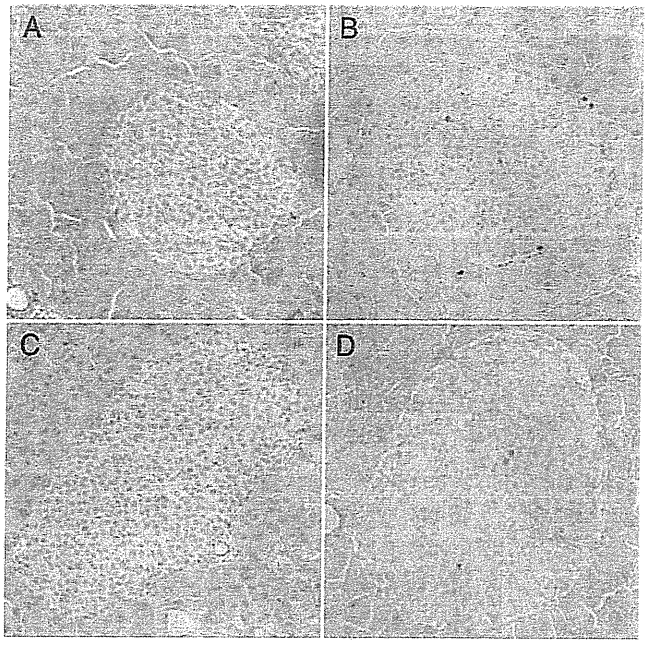

FIG. 4. Immunohistochemical staining of BrdU in pancreas sections. Mice were injected with BrdU 12 h before being killed. BrdU-positive cells (*brown*) were observed in the islets of both WT (B) and SMP30/GNL KO mice (D) on the HFD. A and C, WT (A) and SMP30/GNL KO mice (C) on the SD. Magnification, ×100.

Insulin secretion and ATP measurements from isolated islets

To confirm β-cell dysfunction in SMP30/GNL KO mice, we measured insulin secretion in response to glucose and KCl from the isolated islets. The basal insulin secretion in SD-fed SMP30/GNL KO and WT mice were 89.3 ± 9.8 and 93.3 ± 10.4 pg/islet · 15 min (n = 10), respectively. There were no significant differences between the two. Similar to *in vivo* results, insulin secretion of SD-fed SMP30/GNL KO mice in 20 mM glucose for 15 min was significantly reduced compared with those of SD-fed WT mice ($P < 0.05$, Fig. 5A). Insulin secretory response to 20

TABLE 2. Morphometric analysis and insulin contents of pancreases after 8 wk of SD or HFD feeding

	Pancreas weight (mg/g body weight), n = 10	Pancreatic insulin content (μg/g pancreas), n = 10	β-Cell mass (mg), n = 5	BrdU index (%), n = 5
SD				
WT	5.4 ± 0.4	42.1 ± 3.9	1.21 ± 0.21	4.4 ± 2.8
SMP30/GNL KO	4.1 ± 0.2[a]	40.4 ± 4.6	1.14 ± 0.13	6.9 ± 3.5
HFD				
WT	5.4 ± 0.2	56.7 ± 4.9[b]	3.28 ± 0.85[b]	37.3 ± 11.7[b]
SMP30/GNL KO	5.6 ± 0.4	53.1 ± 4.1[b]	2.83 ± 0.41[b]	47.3 ± 10.6[b]

BrdU index: total BrdU positive islet cells/total islet counts per section.

[a] $P < 0.05$ *vs.* the other three groups.

[b] $P < 0.05$ *vs.* SD-fed WT and SMP30/GNL KO mice.

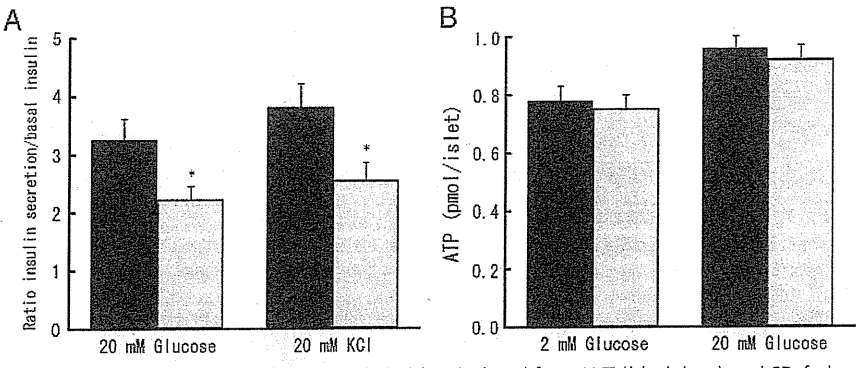

FIG. 5. Insulin secretion and ATP content in islets isolated from WT (*black bars*) and SD-fed SMP30/GNL KO mice (*gray bars*) (14–15 wk of age). A, Static islet incubation for 15 min in 20 mM glucose or 20 mM KCl. The data are expressed as ratio of 15 min to basal insulin (picograms per islet per 15min). *, $P < 0.05$ *vs.* WT mice. Data are means ± SE (n = 5 per group). B, ATP content at 2 and 20 mM glucose. There were no significant differences in ATP content at 2 or 20 mM glucose between WT and SMP30/GNL KO mice. Data are means ± SE (n = 5 per group).

mM KCl was also reduced significantly in SMP30/GNL KO mice ($P < 0.05$, Fig. 5A).

Islet ATP content was significantly elevated at 20 mM glucose compared with 2 mM glucose in SD-fed SMP30/GNL KO and WT mice. However, there were no significant differences in ATP content at 2 or 20 mM glucose between the two (Fig. 5B).

Discussion

This is the first report documenting impaired glucose tolerance in SMP30/GNL-deficient mice and provides new insight into the possible role of SMP30/GNL in glucose homeostasis. Our *in vitro* data using isolated islets confirmed that deletion of SMP30/GNL was responsible for the reduced insulin secretory response to glucose. The present study differs from previous studies using SMP30/GNL KO mice, in that the mice were maintained with a sufficient supply of vitamin C. This eliminated the confounding secondary effects of vitamin C on glucose homeostasis and made this experimental model more relevant to human disease.

SMP30/GNL KO mice at 15 wk of age demonstrated mild glucose intolerance compared with WT mice. They also showed an impairment in acute insulin secretion after glucose administration and better peripheral insulin sensitivity as assessed by an insulin tolerance test. These data suggest that an impairment of the early phase of insulin secretion underlies the glucose intolerance seen in SMP30/GNL KO mice. In HFD-fed WT mice as well as in SMP30/GNL KO mice, HFD feeding increased fat mass and worsened glucose tolerance. However, this impairment of glucose tolerance was more pronounced in SMP30/GNL KO mice. This difference likely reflects SMP30/GNL KO mice's intrinsic reduction in insulin secretion capacity, as shown by their impaired acute insulin secretion after a glucose load and similar insulin resistance to WT mice when fed an HFD. It has been reported that vitamin C treatment has no effect on insulin secretion in diabetic C57BL/KsJ-db/db mice or nondiabetic control mice (21). Thus, the differential vitamin C status seen in this study is unlikely to be the cause of the impairment of insulin secretion.

It is interesting that SMP30/GNL KO mice had the higher peripheral insulin sensitivity. The better insulin sensitivity might partially compensate for the decreased insulin secretion and made the moderate impairment of glucose tolerance. Although the present data could not refer to its mechanism, the lower epididymal and sc fat mass in SMP30/GNL KO mice may help account for the result. Now, we can just assume that absence of SMP30/GNL in insulin-sensitive tissues, including liver, kidney, fat, and muscle, may directly affect insulin signaling pathway or secondarily affect insulin action through metabolic pathways. Also, an increased insulin clearance from the liver is another possibility. It is well known that increased insulin resistance is a major factor involved in impaired glucose tolerance in the elderly. So we would say that SMP30/GNL KO mice is not an appropriate animal model of impaired glucose tolerance in normal aging.

The static incubation study of islets demonstrated the reduced insulin secretory response to glucose and KCl in SMP30/GNL-deficient mice. KCl depolarizes the β-cell plasma membrane, which initiates a series of events such as Ca^{2+} influx, mobilization of Ca^{2+} from intracellular stores, and insulin exocytosis. On the other hand, glucose-stimulated insulin secretion from the β-cell occurs after generation of ATP from metabolism of glucose through glycolysis and the Krebs cycle. The intracellular rise in ATP/ADP ratio leads to closure of the K_{ATP} channels, Ca^{2+} influx, and subsequent activation of insulin secretion. The reduced insulin secretory response to KCl, together with the preservation of ATP production, suggests that events in the distal portion of the insulin secretion pathway are impaired in SMP30/GNL-deficient mice. The mechanism underlying β-cell dysfunction in SMP30/GNL KO mice has not been fully explored. However, previous reports have proposed the possibility of dysregulation of Ca^{2+} homeostasis. SMP30/GNL maintains Ca^{2+} homeostasis by enhancing plasma membrane Ca^{2+}-pumping activity (3, 4). Therefore, taken together with the present results of *in vitro* islet study, a deficiency of SMP30/GNL

may induce dysregulation of Ca^{2+} homeostasis, resulting in impairment of the increase in Ca^{2+} influx resulting from a high glucose concentration and of Ca^{2+}-dependent signaling pathways, which are involved in the mechanisms of insulin secretion. Additional studies, such as intracellular calcium levels in β-cells may allow for a more precise localization of these abnormalities.

It has been well documented that HFD feeding induces compensatory β-cell hyperplasia in response to insulin resistance in mice (22, 23). In the present study, too, HFD feeding caused insulin resistance in both WT and SMP30/GNL KO mice, but there were no differences in the degree of compensatory increase in β-cell mass and proliferation between WT and SMP30/GNL KO mice. These findings suggest that SMP30/GNL deficiency causes β-cell dysfunction without impairment of the mechanism responsible for islet hyperplasia.

Both decreased insulin secretion and increased insulin resistance are two major factors involved in impaired glucose tolerance in the elderly. Increased adiposity and decreased physical activity contribute to insulin resistance in the elderly (15). Recent reports indicate that the age-associated decline in muscle mitochondrial function is a mechanism for insulin resistance (24). On the other hand, the molecular mechanism for the development of age-dependent β-cell defects is still unknown. Aging could be associated with a loss of β-cell mass or impaired β-cell function or a combination of these two factors (25–27). The present results suggest the possibility that a reduction in SMP30/GNL with age contributes to the age-related impairment of β-cell function. If this is the case, SMP30/GNL could be a novel molecule that is involved not only in the impaired insulin secretion that occurs with normal aging but also in the pathogenesis of type 2 diabetes.

In summary, SMP30/GNL KO mice have modestly impaired glucose tolerance with an impairment of acute insulin secretion due to dysfunction of the distal portion of the secretion pathway. The increase in β-cell mass and proliferation to compensate for insulin resistance is equivalent to that of WT mice. The present results suggest that reduction in SMP30/GNL could be a factor that contributes to the worsening of glucose tolerance with normal aging and that this mechanism may provide new insights into the age-associated pathophysiology of β-cell function.

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Developmental and Age-Related Changes of Peptidylarginine Deiminase 2 in the Mouse Brain

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Peptidylarginine deiminases (PADs) are a group of posttranslational modification enzymes that citrullinate (deiminate) protein arginine residues in a Ca^{2+}-dependent manner. Enzymatic citrullination abolishes positive charges of native protein molecules, inevitably causing significant alterations in their structure and functions. Among the five isoforms of PADs, PAD2 and PAD4 are proved occupants of the central nervous system (CNS), and especially PAD2 is a main PAD enzyme expressed in the CNS. We previously reported that abnormal protein citrullination by PAD2 has been closely associated with the pathogenesis of neurodegenerative disorders such as Alzheimer's disease and prion disease. Protein citrullination in these patients is thought to play a role during the initiation and/or progression of disease. However, the contribution of changes in PAD2 levels, and consequent citrullination, during developmental and aging processes remained unclear. Therefore, we used quantitative real-time RT-PCR, Western blot analysis, and immunohistochemical methods to measure PAD2 expression and localization in the brain during those processes. PAD2 mRNA expression was detected in the brains of mice as early as embryonic day 15, and its expression in cerebral cortex, hippocampus, and cerebellum increased significantly as the animals aged from 3 to 30 months old. No citrullinated proteins were detected during that period. Moreover, we found here, for the first time, that PAD2 localized specifically in the neuronal cells of the cerebral cortex and Purkinje cells of the cerebellum. These findings indicate that, despite PAD2's normally inactive status, it becomes active and citrullinates cellular proteins, but only when the intracellular Ca^{2+} balance is upset during neurodegenerative changes.

Key words: cerebellum; citrullination; neurodegenerative disorder; PAD2; Purkinje cells

Because concentrations of peptidylarginine deiminases (PADs; EC 3.5.3.15) become altered during developmental and aging processes and have been linked to abnormal accumulations of citrullinated proteins in degenerative diseases of the brain, we sought to establish in detail the levels and impact of PADs in neonatal and aging mice. PADs are posttranslational modification enzymes that citrullinate (deiminate) protein arginine residues in a calcium ion-dependent manner, yielding citrulline residues (Watanabe et al., 1988; Vossenaar et al., 2003). Enzymatic citrullination abolishes positive charges of native protein molecules, thereby altering their structure and functions over time (Tarcsa et al., 1996). Although the five isoforms of PADs (i.e., types 1, 2, 3, 4/5, and 6) reside in multiple mammalian tissues (Watanabe et al., 1988; Vossenaar et al., 2003), their tissue-specific expression differs according to analysis by reverse transcriptase-polymerase chain reaction (RT-PCR; Ishigami et al., 2001). However, all these isoforms display nearly identical amino acid sequences (Ishigami et al., 2002). Among them, PAD2 and PAD4 occupy the central nervous system (CNS), and especially PAD2 is a main PAD enzyme expressed in the CNS.

These isoforms are present in myelin sheath, and hypercitrullination of myelin basic protein (MBP) resulted in loss of myelin sheath integrity in multiple sclerosis (MS)

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patients (Moscarello et al., 1994; Musse et al., 2008; Wood et al., 2008). Moreover, PAD4, the nuclear isoform of this family of enzymes, is involved in histone citrullination in the MS brain (Mastronardi et al., 2006). Immunocytochemical studies have localized PAD2 in glial cells, especially astrocytes (Asaga and Ishigami, 2000, 2001), microglial cells (Asaga et al., 2002), and oligodendrocytes (Akiyama et al., 1999). Additionally, PAD2 expression was later detected in cultured Schwann cells (Keilhoff et al., 2008). However, although the presence of PAD2 in glial cells should have imbued them with citrullinated proteins, such proteins were rarely identified in those cells examined with our sensitive detection method (Senshu et al., 1992). Therefore, we assumed that PAD2 is normally inactive (Asaga and Ishigami, 2000, 2001; Asaga et al., 2002). However, under abnormal conditions, glial fibrillary acidic protein (GFAP) was highly susceptible to the attack of PAD2 in excised rat brains deliberately left at room temperature (Asaga and Senshu, 1993). Under hypoxic conditions (Asaga and Ishigami, 2000) and during kainic acid-evoked neurodegeneration (Asaga and Ishigami, 2001; Asaga et al., 2002), PAD2 became activated in regions undergoing neurodegeneration and functioned to citrullinate various cerebral proteins, indicating the involvement of protein citrullination in neurodegenerative processes. These findings provided an important clue; that is, PAD2 normally remains inactive but becomes active and citrullinates cellular proteins only when the intracellular calcium balance is upset during neurodegenerative changes. In fact, our previous report indicated that citrullinated proteins including GFAP, vimentin, and myelin basic protein (MBP) accumulated to an abnormal extent in the Alzheimer's disease (AD)-afflicted hippocampus and that the expression of PAD2 increased during related neurodegenerative changes (Ishigami et al., 2005). We subsequently described increases of the citrullinated proteins GFAP, MBP, enolases, and aldolases in the brains of mice infected with scrapie as a model of prion disease along with the increased expression and activity of PAD2, suggesting that accumulated citrullinated proteins and abnormal activation of PAD2 may play a role in the pathogenesis of prion diseases. Moreover, in patients with MS, citrullinated MBP was increased to 45% of total MBP compared with much smaller amounts in healthy adults (Moscarello et al., 1994) and was later regarded as a pathological mechanism of MS (Moscarello et al., 2007).

Because PAD2 activation and accumulation of citrullinated proteins in the brain are increasingly associated with the progression of neurodegenerative disorders such as AD, prion disease, and MS, it is very important to clarify the expression and localization of PAD2 in the brain during the developmental and aging process. For comparison of PAD2 expression and characteristic localization, we used microtubule-associated protein 2 (MAP2) and neurofilament 3 (Nef3) as neuronal cell markers and GFAP as a marker of astrocyte in this study. After applying quantitative real-time RT-PCR, Western blot analysis, and immunohistochemical methods, we found that PAD2 mRNA expression levels increased significantly in the cerebral cortex, hippocampus, and cerebellum during aging. Moreover, for the first time, PAD2 was identified in neuronal cells of the cerebral cortex and Purkinje cells of the cerebellum.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice 3, 6, 12, 24, or 30 months of age were obtained from the Animal Facility at Tokyo Metropolitan Institute of Gerontology, and pregnant female mice were obtained from Japan SLC (Shizuoka, Japan). Mice at the embryonic days 15, 16, 17, and 18; postnatal days 1, 2, 3, 7, and 30; and ages 3, 6, 12, 24, and 30 months were used in this study. Throughout the experiments, animals were maintained on 12-hr light/dark cycles in a controlled environment. All experimental procedures using laboratory animals were approved by the Animal Care and Use Committee of Tokyo Metropolitan Institute of Gerontology.

Brain Sample Preparation

Mice were anesthetized and systemically perfused with phosphate-buffered saline to wash out blood cells. Their brains were quickly excised and divided into cerebral cortex, hippocampus, and cerebellum. Brain sections were homogenized in 10 mM Tris-HCl (pH 7.6) containing 1 mM phenylmethylsulfonyl fluoride and centrifuged at 21,000*g* for 10 min at 4°C. For Western blot analysis, the supernatants were boiled for 5 min with a lysis buffer containing 0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.2% bromophenol blue in a ratio of 1:1 and kept at −80°C until use. The protein concentration was determined by BCA protein assay (Pierce Biotechnology, Rockford, IL) using bovine serum albumin as a standard. To prepare total RNA, the brain sections were immediately frozen in liquid nitrogen and stored at −80°C until use. For immunohistochemical staining, brain tissues were immersed in 10% formalin (Wako Pure Chemical, Osaka, Japan) and left standing for 48 hr. Each fixed brain was cut in half laterally, then embedded in paraffin, and finally cut serially into 6-μm-thick cryosections. Human brain samples were obtained from the Brain Bank for Aging Research (BBAR) organized by the Tokyo Metropolitan Geriatric Hospital and Tokyo Metropolitan Institute of Gerontology (TMIG). Human studies were approved by the Ethics Committees of the TMIG.

PAD2 Monoclonal Antibody

The entire coding sequence of human PAD2 (hPAD2; GenBank AB030176) was amplified by PCR using primers, pENTR-hPAD2 forward primer 5′-CACCATGCTGCGCGAGCGGACCGTGCGGCTG-3′ and pENTR-hPAD2 reverse primer 5′-CCGGAATTCGCGGCCGCTCTGGGCGTGTGAGGGAGGGTCTGGAG-3′. The PCR products were subcloned in pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA). To produce N-terminal 6× his-tagged hPAD2-recombinant protein, hPAD2 cDNA was subcloned to pDEST17 vector (Invitrogen) and transformed with the BL21-AI strain of *Escherichia coli*. The transformants were grown overnight, then treated for 4 hr with 0.2% L-arabinose at 37°C. The bacteria were then disrupted by sonication, and the recombinant proteins were purified by using Ni-NTA

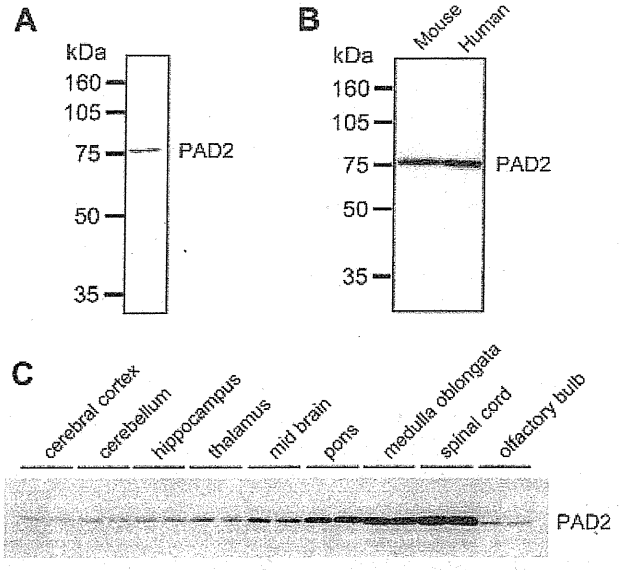

Fig. 1. Characterization of the anti-hPAD2 monoclonal antibody hPAD2-2110 and distribution of PAD2 protein in the CNS. **A:** Purified recombinant hPAD2 protein was separated on 10% SDS-PAGE, followed by Coomassie brilliant blue staining. **B:** Mouse and human brain samples were prepared as described in Materials and Methods. Ten micrograms of extracted proteins were separated on 10% SDS-PAGE and then electrotransferred onto a PVDF membrane. PAD2 was detected by using anti-hPAD2 monoclonal antibody. **C:** Under the stereoscopic microscope, each brain from 3-month-old mice was divided into nine parts: the cerebral cortex, cerebellum, hippocampus, thalamus, midbrain, pons, medulla oblongata, spinal cord, and olfactory bulb. From each part, 10 μg of extracted proteins was separated on 10% SDS-PAGE. PAD2 was detected by using anti-hPAD2 monoclonal antibody. Analyses were performed in duplicate with samples from two individual mice.

agarose beads (Qiagen, Valencia, CA). The identity of recombinant hPAD2 proteins was verified by sodium dodecyl sulfate (SDS)- polyacrylamide gel electrophoresis (PAGE), followed by Coomassie brilliant blue staining (see Fig. 1A).

Purified recombinant hPAD2 (50 μg) in complete Freund's adjuvant was injected into BALB/c mice, which were then given a booster injection of the same antigen in incomplete Freund's adjuvant. Three days after the last injection, spleen cells were fused with mouse P3U1 myeloma cells by using polyethylene glycol, and fused cells were cultured with HAT medium (Invitrogen). The specificities of hPAD2 monoclonal antibody-producing cells, hPAD2-264, hPAD2-2110, hPAD2-2111, hPAD2-2147, hPAD2-2153, hPAD2-2167 clones, were determined by enzyme-linked immunosorbent assays and Western blot analysis. Only the anti-hPAD2 monoclonal antibody hPAD2-2110 was purified from ascites by protein G Sepharose 4 Fast Flow columns (GE Healthcare, Piscataway, NJ). Anti-hPAD2 monoclonal antibody hPAD2-2110 reacted with both human and mouse PAD2 in the brain as confirmed by Western blot analysis (Fig. 1B).

Western Blot Analysis

Equal amounts of protein (10 μg/lane) were separated by SDS-PAGE on vertical slab 10% polyacrylamide gels (1 mm × 9 cm) by the method of Laemmli (1970). Proteins were then electrophoretically transferred from polyacrylamide gels onto a membrane of poylvinylidene difluoride (PVDF; Millipore, Billerica, MA) by the method of Towbin et al. (1979). The membrane was then incubated successively with anti-hPAD2 monoclonal antibody (hPAD2-2110; 1:1,000) and horseradish peroxidase-labeled goat anti-mouse IgG (Bio-Rad, Hercules, CA). Chemiluminescence signals were detected with a LAS-3000 imaging system (Fujifilm, Tokyo, Japan) using ECL Western Blotting Detection Reagents (GE Healthcare UK Ltd. Amersham, Little Chalfont, Buckinghamshire, United Kingdom). Signal intensity was analyzed by using Multi Gauge software (Fujifilm).

RT-PCR

Total RNA was extracted by using Isogen (Wako Pure Chemical, Osaka, Japan). Brain samples were homogenized with a Teflon-pestle homogenizer in Isogen, and total RNA was extracted according to the supplier's instructions. The final RNA pellet was dissolved in diethyl pyrocarbonate-treated H_2O, and the RNA concentration was determined and confirmed to be free from protein contamination by measuring absorbance at 260 and 280 nm. Five micrograms of total RNA from each sample was treated with Turbo DNase I (Ambion, Austin, TX) to eliminate any trace of genomic DNA. RT-PCR was performed with a Takara mRNA Selective PCR Kit (Takara, Kyoto, Japan) according to the supplier's instructions. Reverse transcription was performed with antisense oligonucleotide primers. Mouse PAD2 primers were designed in the 3′ noncording region of the cDNA after checking the absence of homology with any other PAD sequences by using the Blast program. The sense and antisense primers used were, for mouse PAD2, 5′-CTGCGG TCTCTGGGTCCTTCCTGTA-3′ and 5′-GACCAGGC-GAGAGAACAGAAATAGC-3′ (expected size 665 bp; Watanabe and Senshu, 1989); for mouse GFAP, 5′-CTGGAGG TGGAGAGGGACAACTT-3′ and 5′-CCGCATCTCCA-CAGTCTTTACCA-3′ (expected size 840 bp; Balcarek and Cowan, 1985); for mouse MAP2, 5′-GTGAACAAGA-GAAGGAAGCCCAACA-3′ and 5′-GGACCTGCTTGGG-GACTGTGTGATG-3′ (expected size 956 bp; Lewis et al., 1988); and, for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-GTGAAGGTCGGTGTGAACGGAT-3′ and 5′-GCCGCCTGCTTCACCACCTTCTT-3′ (expected size 788 bp; Tso et al., 1985). PCR conditions were 30 sec at 94°C, followed by 25, 30, or 40 cycles at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min, and a terminal extension period (72°C, 5 min). PCR products were visualized by gel electrophoresis in 1.2% agarose with ethidium bromide staining. To confirm their identity, PCR products were subcloned and sequenced.

Quantitative Real-Time PCR Analysis

A final preparation of 1.8 μg for each total RNA was subjected to two-step quantitative real-time PCR. In the first step, reverse transcription reaction was carried out with a random primer and SuperScript II (Invitrogen) in the presence of RNase inhibitor. For the second step, synthesized cDNA was

applied to the inventoried TaqMan gene expression assay by using the real-time PCR equipment (Applied Biosystems 7300 Real Time PCR System; Applied Biosystems, Foster City, CA). For quantitative analysis, a standard curve method was designed using a common standard prepared by mixing an aliquot of all samples from the experiment, and the standard was diluted serially to compute the threshold cycle covering the range between 18 and 35. The samples were also diluted properly for each of the target genes and GAPDH to be measured on the linear range of the semilogarithmic standard curve. All assays were performed under the standard curve correlation factor above 0.990. Values of unknown samples were corrected for dilution factor and normalized to the GAPDH level assumed as a constant. The expression of PAD2, GFAP, and Nef3 was analyzed, and the results are shown as percentage value; i.e., the level at postnatal day 30 and hippocampus at 3-months-old was considered as 100%. All assays were performed in duplicate.

Immunohistochemical Staining

After removal of paraffin, the brain sections were heated by microwave in 0.1 M citrate buffer (pH 7.0), followed by inactivation of endogenous peroxidase during incubation with 1% hydrogen peroxide in methanol. The primary antibodies used were mouse monoclonal PAD2 antibody (hPAD2-2110, 1:1,000), rabbit polyclonal GFAP antibody (1:1,000; Cosmo Bio, Tokyo, Japan), and mouse monoclonal MAP2 antibody (1:1,000; Chemicon, Temecula, CA). PAD2, GFAP, and MAP2 were detected by indirect immunoperoxidase staining using corresponding Histofine Simple Stain MAX-PO kits (Nichirei Biosciences, Tokyo, Japan) and 3,3′-diaminobenzidine (DAB) as a chromogenic substrate. After DAB staining, nuclei were counterstained with Myer's hematoxylin.

For double staining of PAD2 and calbindin-D-28K (calbindin), rabbit anticalbindin antibody (Thermo Fisher Scientific, Fremont, CA) was reacted first, and the alkaline phosphatase-conjugated anti-rabbit IgG (Histofine; Nichirei Biosciences) was reacted to stain with a Red Alkaline Phosphate Substrate Kit I. Second, anti-PAD2 monoclonal antibody (hPAD2-2110) was reacted, then horseradish peroxidase-conjugated anti-mouse IgG was reacted to stain with DAB.

Statistical Analysis

Results are expressed as mean ± SEM. The probability of statistical differences between experimental groups was determined by Bonferroni's multiple-comparisons test subsequent to one-way ANOVA. A statistical difference was considered significant at $P < 0.05$.

RESULTS

Distribution of PAD2 Protein in the CNS

We searched for PAD2 protein throughout the CNS by examining the cerebral cortex, cerebellum, hippocampus, thalamus, midbrain, pons, medulla oblongata, spinal cord, and olfactory bulb of 3-month-old mice. Western blot analysis revealed PAD2 protein in all these regions (Fig. 1C). Specifically, we calculated a ratio of PAD2 protein in the cerebellum, hippocampus, thalamus, midbrain, pons, medulla oblongata, spinal cord, and olfactory bulb compared with the amount of PAD2 protein in the cerebral cortex as 1.3-fold, 1.4-fold, 1.7-fold, 2.3-fold, 3.8-fold, 5.6-fold, 9.9-fold, and 1.6-fold, respectively.

Developmental Change of PAD2 mRNA Expression Level in the Whole Brain

To assess our assumption that PAD2 mRNA expression levels undergo developmental changes, we examined whole brains of mice from embryonic day 15 to postnatal day 30 by both RT-PCR and quantitative real-time PCR. Small amounts of PAD2 mRNA were detected at 15 days of embryonic life and increased slightly until birth (Fig. 2A,B). After birth, the PAD2 mRNA level was constant until the third postnatal day and then significantly increased 2.5-fold at day 7 and 9.9-fold at day 30 compared with value at postnatal day 3 (Fig. 2B). Moreover, the PAD2 mRNA level at 30 days after birth was a statistically significant 3.9-fold higher than that at the seventh postnatal day. Similarly, amounts of PAD2 protein in the whole brain were small at embryonic day 16, held constant until the third postnatal day, then significantly increased to 5.9-fold at 7 days and to 17-fold at 30 days after birth compared with the amount of PAD2 protein at the third postnatal day (Western blot analysis; Fig. 3).

GFAP as a marker of astrocyte mRNA was detected at 16 days of embryonic life and increased until 3 postnatal days, then continued to increase until reaching a significant increment at the seventh and again at the thirtieth postnatal days (Fig. 2A,C). The GFAP mRNA level at 7 days and 30 days after birth also rose significantly to 3.4-fold and 2.4-fold higher levels than that at 3 days after birth (Fig. 2C). On the other hand, mRNAs for MAP2 and Nef3, the neuronal cell markers, were detectable in 15-day-old embryos and increased slightly until birth (Fig. 2A,D). The Nef3 mRNA level at 30 days of postnatal was a significant 1.3-fold higher than that at postnatal day 7 (Fig. 2D).

Age-Related Change of PAD2 mRNA Expression Level

The age-related changes of PAD2, GFAP, and Nef3 mRNA in the cerebral cortex, cerebellum, and hippocampus of 3-month-old to 30-month-old mice were analyzed by quantitative real-time PCR. PAD2 mRNA expression was detected in the cerebral cortex, cerebellum, and hippocampus, but the cerebral cortex contained significantly less PAD2 mRNA than was found in the cerebellum and hippocampus regardless of the animals' ages (Fig. 4A). Moreover, PAD2 mRNA levels at all three sites gradually increased during aging. PAD2 mRNA levels in the cerebral cortex, cerebellum, and hippocampus at 30 months were about 1.6-fold, 1.5-fold, and 1.6-fold higher, respectively, than in 3-month-old animals.

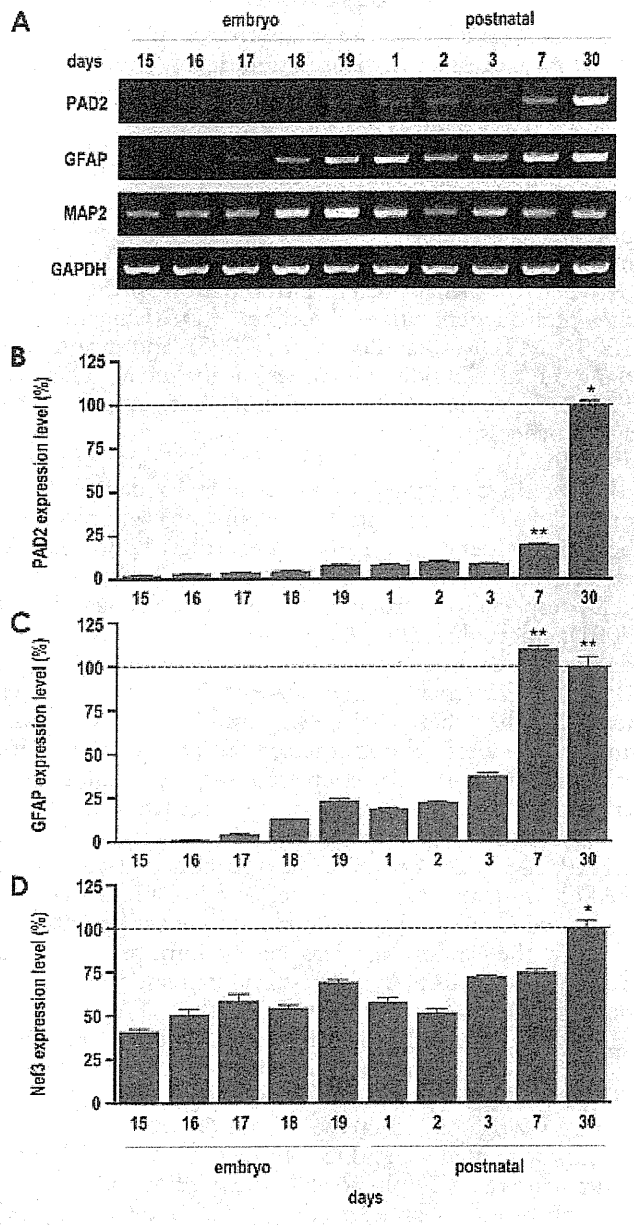

Fig. 2. Developmental changes of PAD2, GFAP, MAP2, and Nef3 mRNA expression levels in the whole brains of mice. **A:** RT-PCR was carried out with 1 μg of total RNA and specific oligonucleotide primer of PAD2, GFAP, MAP2, and GAPDH as described in Materials and Methods. An aliquot of each PCR product was electrophoresed in 1.2% agarose gel and stained with ethidium bromide for detection under UV light. **B–D:** Quantitative real-time PCR analysis was carried out with TaqMan primers and probes specific to PAD2 (B), GFAP (C), and Nef3 (D). As the endogenous control, GAPDH was quantified simultaneously and used to normalize each raw data point. Data from quantitative real-time PCR are shown as the percentage of each value, with postnatal day 30 taken as 100%, and represent mean ± SEM of five animals. $*P < 0.05$ compared with 15–19 days of embryonic life and 1–3 or 7 days after birth. $**P < 0.05$ compared with 15–19 days of embryonic life and 1–3 days after birth.

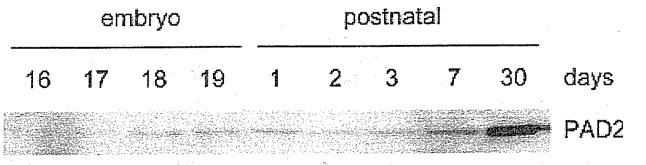

Fig. 3. Western blot analysis of PAD2 protein in the whole brain during development from embryonic day 16 to postnatal day 30. Ten micrograms of protein extracted from the whole brain was separated on 10% SDS-PAGE and then electrotransferred onto the PVDF membrane. PAD2 was detected by using anti-hPAD2 monoclonal antibody, hPAD2-2110.

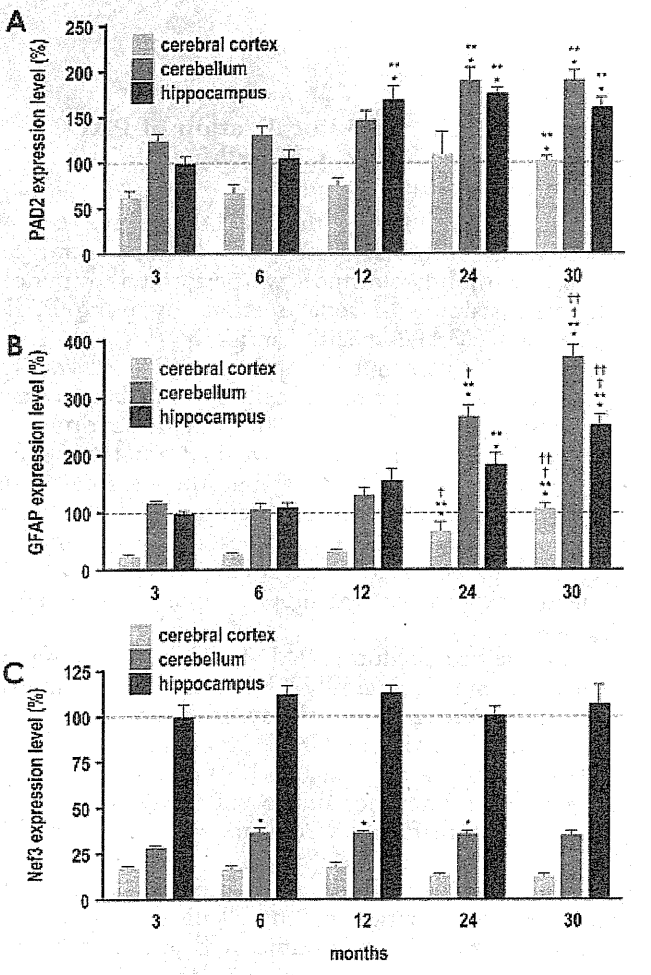

Fig. 4. Age-dependent changes of PAD2, GFAP, and Nef3 mRNA expression in the brain. Total RNA from the cerebral cortex, cerebellum, and hippocampus of 3-, 6-, 12-, 24-, and 30-month-old mice was prepared. Quantitative real-time PCR analysis of PAD2 (**A**), GFAP (**B**), and Nef3 (**C**) was carried out as described in Materials and Methods. As the endogenous control, GAPDH was quantified simultaneously to normalize each raw data. Data are expressed in percentages, with values in the hippocampus of 3-month-old mice taken as 100%, and represent a mean ± SEM of five animals. $*P < 0.05$ compared with 3-month-old mice. $**P < 0.05$ compared with 6-month-old mice. $^{\dagger}P < 0.05$ compared with 12-month-old mice. $\dagger\dagger P < 0.05$ compared with 24-month-old mice.