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6 Abstract: Here we present the first evidence that muscle-specific kinase (MuSK) antigen  
7 can cause myasthenia in animals. MuSK is expressed at the postsynaptic membranes of  
8 neuromuscular junctions (NMJ) and forms complexes with acetylcholine receptors  
9 (AChR) and rapsyn. MuSK is activated by agrin, which is released from motoneurons,  
10 and induces AChR clustering and subsequent formation of NMJ in embryos. Notably,  
11 autoantibodies against MuSK were found in a proportion of patients with generalized  
12 myasthenia gravis (MG) but without the characteristic AChR autoantibodies. However,  
13 MuSK autoantibodies had no known pathogenic potential, and animals immunized with  
14 purified MuSK proteins did not develop MG in former studies. In contrast, we have  
15 now injected rabbits with MuSK ectodomain protein *in vivo* and evoked a MG-like  
16 muscle weakness with a reduction of AChR clustering at the NMJ. Our results showed  
17 that MuSK is required for maintenance of synapses and that interference with that  
18 function by MuSK antibodies causes myasthenic weakness. *In vitro*, AChR clustering in  
19 myotubes is induced by agrin and agrin-independent inducers, which do not activate  
20 MuSK. Neither the receptor nor the activation mechanisms of AChR clustering induced  
21 by agrin-independent inducers has been identified with certainty, but MuSK  
22 autoantibodies in myasthenic animals inhibited both agrin and agrin-independent AChR  
23 clustering. MuSK plays multiple roles in pre-patterning of the postsynaptic membrane  
24 before innervation and formation of NMJ in embryos. Some of these mechanisms may  
25 also participate in the maintenance of mature NMJ. This model system would provide  
26 new knowledge about the molecular pathogenesis of MG and MuSK functions in  
27 mature NMJ.  
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## INTRODUCTION

Although autoantibodies against muscle-specific kinase (MuSK) have been found in patients with myasthenia gravis (MG)(1), any pathogenic contribution of MuSK antibodies to the muscle weakness that typifies MG has remained in dispute. That is, until now, MuSK antibodies have not produced experimental autoimmune MG (2, 3). Here we describe the recent progress toward understanding this phenomenon.

## AUTOANTIBODIES AGAINST MuSK

About 80% of patients with MG have autoantibodies against AChR. A seminal experiment by Patric and Lindstrom demonstrated the pathogenicity of autoantibodies to AChR about 30 years ago(4). Although a number of studies have documented that AChR antibodies cause structural and functional damage to the neuromuscular junction (NMJ), autoantigens in the nearly 20% of MG patients without such antibodies remained obscure(1). Then, in 2001, Hoch et al. identified antibodies against MuSK in a proportion of patients with generalized MG (1). MuSK is required for clustering of AChR during the formation of NMJ and is expressed predominantly at the postsynaptic membrane in mature NMJ(5, 6). In MuSK knock-out mice, AChRs fail to cluster opposite to growing motoneuron terminals on the surfaces of myotubes(7). Additionally, a case of heteroallelic MuSK mutations that caused the reduction of MuSK expression has been associated with congenital myasthenic syndrome (CMS)(8). Further, the reduction of MuSK expression in rat muscles *in vivo* upon RNA interference induced disassembly of synapses(9). Even though the function of MuSK in mature NMJ is still uncertain, a causal relationship between MuSK autoantibodies and MG has been proposed(1, 10, 11).

Recent studies by Vincent and others showed that the frequency of MuSK antibodies in MG patients who were AChR seronegative (lacked autoantibodies to AChR) varied from 4 to 50%(11-17). We detected MuSK antibodies in 29% of seronegative MG patients but not in any MG patients with AChR antibodies (seropositive MG) or with other autoimmune diseases(16). Previously we identified antibodies against a recombinant MuSK fusion protein with human alkaline

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6 phosphatase (AP) in seropositive MG patients(15) and later revealed that 8.8% of  
7 seropositive MG patients had autoantibodies to AP but not to MuSK(16). We are  
8 currently studying the clinical significance of the autoantibodies to AP in seropositive  
9 MG.  
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13 Clinical features of patients with MG and MuSK antibodies are distinctive. Such  
14 patients often have severe bulbar dysfunctions that can be difficult to treat effectively  
15 with immunosuppressive and immunomodulatory strategies, and atrophy of facial and  
16 tongue muscles is common(12, 13, 18, 19). After the identification of MuSK antibodies  
17 in MG patients, laboratory quantification of these antibodies is now required to confirm  
18 the diagnosis of MG, the appropriate clinical treatment as well as the presence of AChR  
19 antibodies(18, 20, 21).  
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#### 28 **EXPERIMENTAL AUTOIMMUNE MG (EAMG)**

29 Although MuSK antibodies are present in some seronegative MG patients and the  
30 clinical features are distinctive, proving the pathogenicity of MuSK antibodies has been  
31 difficult, because these antibodies did not induce myasthenia in experimental animals.  
32 Formerly, the pathogenicity of AChR antibodies was shown when rabbits injected with  
33 AChR protein purified from electric eels developed muscle weakness and paralysis (4).  
34 Injection of eel AChR protein stimulates the production of antibodies that cross-react  
35 with rabbit AChR at the NMJ. Electrophysiological studies confirmed that the flaccid  
36 paralysis in this animal model resembled that in MG patients. Similarly, EAMG  
37 appeared in other species after immunization with purified AChR protein. In addition,  
38 the antibodies to AChR in human MG patients could passively transfer disease to  
39 mice(22). Therefore, creating an EAMG model induced by MuSK antibodies was  
40 indispensable for proving the pathogenicity of MuSK antibodies and investigating their  
41 pathogenic mechanisms in MG (10, 20, 21).  
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54 To pursue this objective, we recently immunized rabbits with MuSK ectodomain,  
55 which caused myasthenic weakness and produced electromyographic findings that were  
56 compatible with a diagnosis of MG, as shown by Patrick and Lindstrom(23). The  
57 extracellular segment of MuSK comprises five distinct domains, i.e., four  
58 immunoglobulin-like domains and one cysteine-rich region(5, 6). The fusion protein  
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6 expression constructs we generated consisted of mouse MuSK ectodomain with the Fc  
7 region of human IgG1 or His-tag and were used to transfect COS-7 cells(23). The  
8 recombinant MuSK-Fc and MuSK-His proteins secreted were purified by using protein-  
9 A Sepharose and histidine affinity columns, respectively (FIG.1). New Zealand white  
10 rabbits were then immunized with 100 to 400 mg of this purified MuSK recombinant  
11 protein. After three to four injections of MuSK protein, all six rabbits so-treated  
12 manifested flaccid paralysis (FIG.2). Sera from the paretic rabbits contained high titers  
13 of MuSK antibodies that reacted specifically with MuSK molecules as observed by  
14 testing sera from MG patients with MuSK antibodies(1, 24). The paretic rabbits  
15 developed severe muscular exhaustion revealed by histological studies showing  
16 alterations in muscle fibers ranging from subtle to angular atrophy intermingled with  
17 normal muscle. Atrophic changes of this type can result from MG, reduced mechanical  
18 activity of muscles, or cachexia. Repetitive electromyograms of a paretic rabbit were  
19 then done to measure the result of stimulating the retroauricular branch at 20 Hz and  
20 recording responses from the retroauricular muscle. The compound muscle action  
21 potential (CMAP) showed a decremental pattern, consistent with MG(23). However, the  
22 injection of ACh esterase inhibitor did not significantly offset the CMAP decrement or  
23 decrease the symptoms. Importantly, the induction of EAMG by MuSK antibodies is  
24 not limited to rabbits, i.e., we and others have also produced EAMG in mice by  
25 injecting MuSK protein (FIG.2) (25).  
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### AChR CLUSTERING AND STRUCTURE OF NMJ IN RABBITS WITH EAMG AND MUSK ANTIBODIES

50 The clustering of AChR necessary for NMJ formation is completely abolished in  
51 MuSK knock-out mice (7), and AChR clustering at the NMJ is reduced in subjects with  
52 CMS and MuSK mutations(8). In a previous RNA interference experiment, Injection of  
53 double-stranded RNA (dsRNA) targeting MuSK diminishes the expression of MuSK  
54 protein and AChR clusters in rat muscle fibers *in vivo*, whereas dsRNA targeting  
55 nonessential proteins does not have any effect (RNA interference experiment) (9).  
56 Therefore, we examined the expression of AChR at NMJ in soleus muscles of paretic  
57 and normal rabbits by fluorescence microscopy after applying a rhodamine-conjugated  
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6 AChR agonist, a-bungarotoxin. Images of AChR clustering were then recorded by using  
7 a digital camera(23). The sizes and optical densities were measured using NIH image  
8 analysis software with unprocessed digitized NIH images. The results unequivocally  
9 pictured a significantly reduced area and intensity of AChR fluorescence in the paretic  
10 rabbits compared with their normal counterparts. In addition, a structural examination  
11 showed that the size and branching of the NMJ were significantly diminished in paretic  
12 rabbits. Similar changes of NMJ structure were observed in rats with reduced  
13 expression of MuSK evident by RNA interference(9), in a patient with CMS and MuSK  
14 mutations and in mice expressing the missense mutation by electroporation  
15 experiments(8). Our results demonstrated that MuSK antibodies also elicited synaptic  
16 changes in EAMG, including the reduced expression of surface AChR at postsynaptic  
17 membranes of NMJ. Further examination of MuSK knock-out mice disclosed  
18 presynaptic defects in addition to postsynaptic ones(7), indicating that MuSK is also  
19 required for presently unidentified retrograde signals to maintain the presynaptic  
20 structure in mature NMJ.  
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### 34 35 **PATHOGENIC MECHANISMS OF MUSK ANTIBODIES IN AChR** 36 **CLUSTERING AT NMJ** 37

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40 MuSK plays multiple roles in clustering AChR during development of the  
41 postsynaptic membrane of NMJ. Contact of the motor-nerve growth cone with the  
42 muscle induces a narrow, distinct endplate zone in the mid-muscle that is marked by a  
43 high density of AChR clustering(26-29). In this step, agrin released from motoneurons  
44 activates MuSK and redistributes AChR clusters to synaptic sites. However, a direct  
45 physical interaction between MuSK and agrin has so far not been demonstrated despite  
46 many efforts to do so(27). Thus, the mechanisms of MuSK activation and the following  
47 events remain obscure, although a co-receptor of MuSK, co-ligand of agrin or either  
48 post-translational modification of agrin or MuSK have been postulated. Intriguingly,  
49 MuSK is also required for organizing a primary synaptic scaffold to establish the post-  
50 synaptic membrane(30, 31). Preceding muscle innervations, AChR clusters form at the  
51 central regions of muscle fibers, creating an endplate zone that is somewhat broader  
52 than that in innervated muscle. Thus, MuSK is required for pre-patterning of AChR  
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6 clustering in the absence of motor innervation. The scenario of MuSK's roles in the  
7 process is somewhat complicated; possibly an element other than agrin achieves  
8 activation of MuSK and triggers postsynaptic specialization at the NMJ, and/or MuSK  
9 acts as a primary scaffold molecule without activation. The listed pleiotropic roles of  
10 MuSK in AChR clustering at NMJ development could also require the maintenance of  
11 mature NMJ. Studies performed *in vivo* have shown that synaptic AChRs intermingle  
12 completely over a period of ~four days and that many extra-synaptic AChRs are  
13 incorporated into the synapse at the mature NMJ, although the synaptic membrane in  
14 adult muscle appears to be macroscopically stable(32, 33). Therefore, the mechanisms  
15 at play in AChR clustering during NMJ development are also required in mature NMJ  
16 when postsynaptic complexes including AChR and MuSK are dynamically turning over  
17 for maintenance.  
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20 To elucidate the mechanisms of AChR clustering at NMJ, a number of studies were  
21 performed using cultured C2C12 myotubes. Agrin induces clustering of AChR in  
22 C2C12 myotubes following MuSK autophosphorylation(26, 27, 29). This event *in vitro*  
23 represents a major cascade of AChR clustering at the NMJ after innervation by  
24 motoneurons(27, 34-36). Laminin-1 and the *N*-acetylgalactosamine (GalNAc)-specific  
25 lectin *Vicia villosa* agglutinin (VVA-B4), can induce AChR clustering in C2C12  
26 myotubes without activation of MuSK(34, 37-40). Neither the receptor nor the  
27 activation mechanisms of AChR clustering induced by agrin-independent inducers has  
28 been identified with certainty. However, these mechanisms may also play important  
29 roles in the maintenance of NMJs via agrin-independent pathways and in their  
30 formation, as shown by genetic studies(30, 31).  
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33 In their previous study, Hoch et al. observed that the MuSK antibodies of MG  
34 patients inhibited agrin-induced AChR clustering in C2C12 myotubes(1). We also found  
35 that agrin-induced clustering of AChR was strongly blocked in the presence of MuSK  
36 antibodies, whereas absorption of the antibodies with purified MuSK products  
37 prevented this blocking effect(23). Thus, MuSK antibodies were responsible for  
38 inhibiting the formation of agrin-induced AChR clustering. We also perceived that  
39 MuSK-specific antibodies strongly inhibited AChR clustering induced by all known  
40 agrin-independent pathways as well as by agrin itself.  
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## CONCLUSIONS

In our experimental model of myasthenia, MuSK antibodies routinely mediated pathogenesis in rabbits and mice(23, 25). Consequently, we now believe that MuSK antibodies cause MG in patients. However, the pathogenic mechanisms of these antibodies entail multiple events in which MuSK acts as a multifunctional platform from which to regulate synapse formation and maintenance. These are reflected in a diversity of clinical features ranging from typical MG to a multitude of variants (12, 13, 18, 19).

AChR antibodies have been shown to affect neuromuscular transmission by three main mechanisms: (a) binding and activation of complement at the NMJ; (b) accelerated degradation of AChR molecules cross-linked by antibodies (antigenic modulation); (c) functional AChR block (20, 21). Intriguingly, MuSK antibodies in MG patients are mainly of the IgG4 subclass, which does not activate complement(41). Electron microscopic observations of NMJ in the EAMG rabbits demonstrated a significant reduction of synaptic folds but no destruction; thus our EAMG model resembles the phenotypes of MG with MuSK antibodies. MuSK antibodies against compound antigenic determinants on the extracellular domain may elicit pathogenic effects through antigenic modulation and/or restraint of MuSK functions(41), and the consequences of these effects range from a partial to entire loss of MuSK functions.

Recently a new MuSK-interacting cytoplasmic protein, called Dok-7, has been discovered(42). Dok-7 knock-out mice underwent a marked disruption of neuromuscular synaptogenesis that was indistinguishable from the features found in MuSK-deficient mice. Mutations in Dok-7 caused a genetic form of limb-girdle myasthenia (CMS)(43, 44). Some clinical features of these patients resemble the severe type of MG with MuSK antibodies(44); therefore, the EAMG model with MuSK antibodies presented here promises to facilitate resolution of the pathogenic basis of MG and CMS at the molecular level and identification of beneficial treatment strategies against them.

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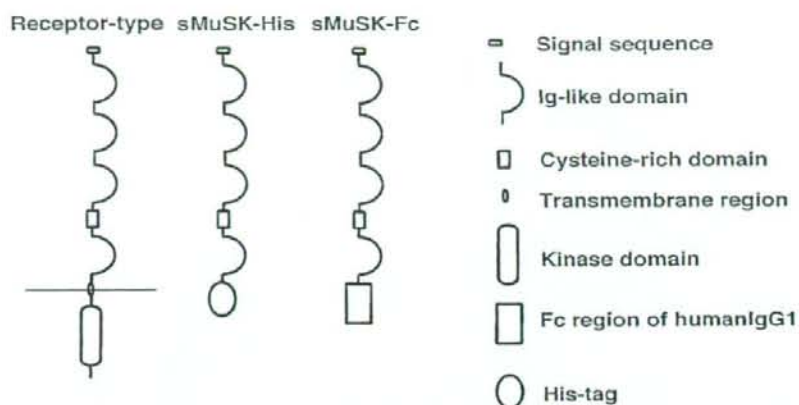
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**FIGURE LEGENDS**

**FIGURE 1.** Schematic representation of the MuSK domain structure and expression of secretory MuSK proteins in COS-7 cells. The domain structures of recombinant secretory MuSK protein (MuSK-His and MuSK-Fc) and receptor-type MuSK are shown. The whole coding region of the MuSK extracellular domain was fused with the His-tag or Fc region of human IgG1 as shown.

**FIGURE 2.** Manifestation of muscle weakness after injection of purified MuSK proteins in experimental animals. Left: a paretic rabbit. Right: a paretic mouse.



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Figure 2. Manifestation of muscle weakness after injection of purified MuSK proteins in experimental animals. Left: a paretic rabbit. Right: a paretic mouse.



## Vitamin C is not essential for carnitine biosynthesis *in vivo*: Verification in vitamin C-depleted SMP30/GNL knockout mice\*

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Carnitine is an essential cofactor in the transport of long-chain fatty acids into the mitochondrial matrix and plays an important role in energy production via  $\beta$ -oxidation. Vitamin C (VC) has long been considered a requirement for the activities of two enzymes in the carnitine biosynthetic pathway, *i.e.*, 6-N-trimethyllysine dioxygenase and  $\gamma$ -butyrobetaine dioxygenase. Our present study using senescence marker protein 30 (SMP30)/gluconolactonase (GNL) knockout (KO) mice, which cannot synthesize VC *in vivo*, led to the conclusion that this notion is not true. After weaning at 40 days of age, SMP30/GNL KO mice were fed a diet lacking VC and carnitine, then given water containing 1.5 g/L VC (VC(+) mice) or no VC (VC(-) mice) for 75 days. Subsequently, total VC and carnitine levels were measured in the cerebrum, cerebellum, liver, kidney, soleus muscle, extensor digitorum longus muscle, heart and plasma. The total VC levels in all tissues and plasma from VC(-) SMP30/GNL KO mice were negligible, *i.e.*, <2% of the levels in SMP30/GNL KO VC(+) mice; however, the total carnitine levels of both groups were similar in all tissues except the heart. In addition, carnitine was produced by incubated liver homogenates from the VC-depleted SMP30/GNL KO mice irrespective of the presence or absence of 1 mM VC. Collectively, these results indicate that VC is not essential for carnitine biosynthesis *in vivo*.

Carnitine (3-hydroxy-4-N-trimethylaminobutyric acid) is a metabolite essential for the transport of long-chain fatty acids from the cytosol into the mitochondrial matrix and is an important player in energy production via  $\beta$ -oxidation (1-3). Therefore, carnitine depletion causes a failure of ATP production and an accumulation of triglycerides in tissues such as the liver, skeletal muscle and heart (4-6). Animal tissues contain relatively large amounts of carnitine with the highest concentrations in heart and skeletal muscle (7). Although animals obtain carnitine primarily from the diet, carnitine is also synthesized by most mammals but is not degraded in the body. Carnitine homeostasis in mammals is maintained by a modest rate of endogenous synthesis, absorption from dietary sources, efficient reabsorption in the kidney and mechanisms present in most tissues that establish and maintain substantial concentration gradients between intracellular and extracellular carnitine pools (8).

Carnitine is synthesized ultimately from the amino acids lysine and methionine (9-14). In some proteins (histones, myosin, calmodulin, and actin), lysine residues are trimethylated on the 4-amino group by specific methyltransferases that use S-adenosyl-L-methionine as the methyl donor (15) (Fig. 1). After lysosomal degradation of these proteins, free 6-N-trimethyllysine (TML) becomes available for carnitine biosynthesis. Four enzymatic steps are required to synthesize carnitine, and the first and last steps are catalyzed by 6-N-trimethyllysine dioxygenase (TMLD, EC

1.14.11.8) and  $\gamma$ -butyrobetaine dioxygenase ( $\gamma$ -BBD, EC 1.14.11.1), respectively. TMLD hydroxylates TML on the 3-position to yield 3-hydroxy-TML (HTML) (16), and  $\gamma$ -BBD hydroxylates  $\gamma$ -butyrobetaine ( $\gamma$ -BB) on the 3-position to yield carnitine (17). Both TMLD and  $\gamma$ -BBD are dioxygenases; hydroxylation of their substrates is coupled to the conversion of 2-oxoglutarate and molecular oxygen to succinate and carbon dioxide. In addition, the TMLD protein shows high homology to the  $\gamma$ -BBD protein, although they appear to belong to separate subfamilies of the 2-oxoglutarate-dependent dioxygenases (3). TMLD is associated predominantly with mitochondria (18,19), whereas  $\gamma$ -BBD is localized in the cytosol (20,21). Although  $\gamma$ -BBD activity has been detected in kidneys from humans, cats, cows, hamsters, rabbits and Rhesus monkeys at equal or higher levels than that in the liver, the activity was not detectable or detected at very low levels in kidneys from Cebus monkeys, sheep, dogs, guinea pigs, mice and rats, in which  $\gamma$ -BBD activity predominates in the liver (22-24).

In 1961, Lindstedt *et al.* (25) first showed that  $\gamma$ -BBD is stimulated considerably by 2-oxoglutarate and that the enzyme requires molecular oxygen, reduced iron ( $Fe^{2+}$ ) and vitamin C (VC, L-ascorbic acid) for enzyme activity. Therefore, many studies reported the enhancement of  $\gamma$ -BBD and TMLD activity upon the addition of VC in a dose-dependent manner using tissue extracts or partially purified enzymes (18-22,26-28). In the absence of VC, however,  $\gamma$ -BBD activity was detected by adding glutathione peroxidase and glutathione (GSH) to the reaction mixture (29), although this test was not performed for TMLD.

To ascertain the necessity of VC for  $\gamma$ -BBD and TMLD activity in carnitine biosynthesis, researchers used guinea pigs that, like humans, cannot synthesize VC *in vivo*. Many reports indicated that carnitine levels, especially in tissues where carnitine is the most abundant such as the heart and skeletal muscle, decreased significantly when the animals became depleted in VC. Since then, VC has been deemed essential for  $\gamma$ -BBD and TMLD activity (30-32). However, in 1990 Alkonyi *et al.* (33) reported that an increase in urinary excretion contributed greatly to a carnitine deficiency in guinea pigs during states of VC

deficiency and starvation. Rebouche (34) also reported that the carnitine depletion related to a VC deficiency results from a decrease in carnitine reabsorption. Thus, since the VC status of such animals influences their urinary excretion of carnitine, guinea pigs are not appropriate subjects for use in studies of carnitine biosynthesis to determine the involvement of VC.

Recently we have established senescence marker protein 30 (SMP30)/gluconolactonase (GNL) knockout (KO) mice, which are incapable of synthesizing VC *in vivo*, because SMP30/GNL is involved in the VC biosynthetic pathway (35). SMP30/GNL KO mice are actually subject to scurvy when fed a VC-deficient diet (35). Therefore, in this study, we used VC-depleted SMP30/GNL KO mice to determine the necessity of VC for carnitine biosynthesis *in vivo* and *in vitro*.

## EXPERIMENTAL PROCEDURES

**Animals.** SMP30/GNL KO mice were previously generated by the gene targeting technique (36). Heterozygous female mice (SMP30/GNL<sup>+/+</sup>) were mated with male KO mice (SMP30/GNL<sup>-/-</sup>) to produce male KO (SMP30/GNL<sup>-/-</sup>) and male wild-type (WT) (SMP30/GNL<sup>+/+</sup>) littermates. Heterozygous male mice do not exist, because the SMP30/GNL gene is located on the X chromosome. Genotypes of SMP30/GNL mutant mice were determined as described previously (36). SMP30/GNL KO and WT mice were weaned at 40 days of age, at which time they were divided into the following four groups: VC [VC(+)], VC-free [VC(-)], WT and SMP30/GNL KO mice. The VC(+) group had free access to water containing VC (1.5 g/L) and 10  $\mu$ M EDTA, whereas the VC(-) group had free access to water without VC. Water bottles were changed every three or four days until the experiment ended. After weaning, all mice were fed a VC- and carnitine-deficient diet (CLEA-purified diet; CLEA Japan, Tokyo, Japan), the composition of which is listed in Table 1. Throughout the experiments, animals were maintained on a 12-h light/dark cycle in a controlled environment. All experimental procedures using laboratory animals were approved by the Animal Care and Use Committee of the Tokyo Metropolitan Institute of Gerontology.

*Measurement of total VC.* L-ascorbic acid in tissues and plasma was measured by a high-performance liquid chromatography (HPLC)-electrochemical detection (ECD) method. Tissues were homogenized in 14 volumes of 5.4% metaphosphate and centrifuged at 21,000 g for 10 min at 4°C. Plasma was mixed with nine volumes of 20% metaphosphate and centrifuged at 21,000 g for 10 min at 4°C. The supernatants obtained were kept at -80°C until use. To measure total VC level, samples were treated with 0.1% dithiothreitol (DTT) to reduce dehydroascorbic acid (DHA) to L-ascorbic acid (37,38). DTT-treated and untreated samples were analyzed by HPLC using an Atlantis dC18 5 µm column (4.6 × 150 mm, Nihon Waters, Tokyo, Japan). The mobile phase was 50 mM phosphate buffer (pH 2.8), 0.2 g/L EDTA, 2% methanol at flow rate of 1.3 ml/min, and electrical signals were recorded by using an electrochemical detector with a glassy carbon electrode at 1.1 V (37,38). DHA levels in samples were calculated from values for DTT-treated minus untreated samples.

*Measurement of total carnitine.* Tissues were homogenized in 100 volumes of 0.14 M NaCl and centrifuged at 9,600 g for 10 min at 4°C. Serum was centrifuged at 9,600 g for 10 min at 4°C. The supernatants obtained after centrifugation were kept at -80°C until use. The total carnitine (acyl carnitine plus free carnitine) levels in tissues and serum were measured by using an enzyme cycling method with carnitine dehydrogenase (39). For this, Total Carnitine Kainos (Kainos Laboratories, Tokyo, Japan) was used.

*Measurement of carnitine urinary excretion.* For measurement of carnitine excreted into urine, a mouse was housed in a metabolic cage, and urine was collected for 24 h in a bottle containing mineral oil to prevent evaporation. This urine was centrifuged at 21,000 g for 10 min at 4°C and kept at -80°C until use. The total carnitine (acyl carnitine plus free carnitine) levels in urine were measured using Total Carnitine Kainos. Creatinin levels in urine were measured using a Creatinin Test Wako kit (Wako Pure Chemical, Osaka, Japan) according to the manufacturer's instruction, and carnitine levels were normalized by creatinin value.

*In vitro carnitine biosynthesis assay.* After SMP30/GNL KO mice were weaned at 40 days of age, they were fed a carnitine- and VC-deficient diet and water without VC for 75 days. These mice were then sacrificed; their livers and kidneys were collected and homogenized in 10 mM Tris-HCl (pH 7.6) containing 1 mM phenylmethylsulfonyl fluoride. For the carnitine biosynthesis assay, the homogenates were incubated in the presence or absence of 1 mM VC at 37°C for 15, 30, 45, 60, and 90 min. The reaction was stopped by immediate transfer of sample tubes into an ice water bath, and the samples were quickly frozen on dry ice. For the measurement of VC, aliquots of the reaction mixtures were mixed with an equal volume of 10% metaphosphate, and the VC levels were measured by the HPLC-ECD methods described above. For the measurement of carnitine, aliquots of the reaction mixtures were centrifuged at 21,000 g for 10 min at 4°C, and the total carnitine levels in the supernatants were measured by using Total Carnitine Kainos. The protein concentration was determined by the BCA protein assay (Pierce Biotechnology, Inc., Rockford, IL, USA) using bovine serum albumin as a standard.

*Measurement of glutathione (GSH).* The GSH levels in tissues and plasma were measured by HPLC (40). Tissues were homogenized in nine volumes of 1 M perchloric acid (PCA) and centrifuged at 21,000 g for 30 min at 4°C. Plasma samples were mixed with one volume of 1 M PCA and centrifuged at 21,000 g for 10 min at 4°C. The supernatants obtained were kept at -80°C until use. The GSH in samples was analyzed by HPLC, using a Sun Fire column (4.6 × 150 mm, Nihon Waters, Tokyo, Japan). The mobile phase was 0.1 M sodium perchlorate monohydrate, 5% acetonitrile, 0.05% trifluoroacetate at a flow rate of 0.5 ml/min, and the absorbance at 220 nm was recorded.

*Statistical analysis.* Results are expressed as means ± SEM. The probability of statistical differences between experimental groups was determined by Student's *t*-test or ANOVA as appropriate. For one- and two-way ANOVAs, we used KaleidaGraph software (Synergy Software, Reading, PA). Statistical differences were considered significant at  $p < 0.05$ .

## RESULTS

**Body weight change.** To investigate, the effect of VC on growth, we compared one group of SMP30/GNL KO mice fed drinking water containing 1.5 g/L VC [VC(+)] with an identical group given water without VC [VC(-)]. The VC(-) mice initially gained weight to the same degree as the VC(+) mice. However, the mean body weight of VC(-) SMP30/GNL KO mice gradually decreased starting at 40 days after weaning (Fig. 2). The mean body weights of the VC(+) and VC(-) KO mice at 70 days after weaning were  $35.2 \pm 1.7$  g and  $23.9 \pm 1.9$  g, respectively, the weight of VC(-) KO mice being less by 32% than that of VC(+) KO mice. Mean water consumption during the experiment was not significantly different between the two groups, *i.e.*,  $3.6 \pm 0.3$  ml/day/mouse for VC(+) KO mice and  $3.2 \pm 0.5$  ml/day/mouse for VC(-) KO mice. Throughout the experiment, the increase with time in body weight of VC(+) SMP30/GNL KO mice was similar to those of VC(+) and VC(-) WT mice that were tested for comparison.

**Total VC and carnitine levels in tissues after VC depletion.** To examine the VC status in the VC(-) SMP30/GNL KO mice, we determined the quantity of total VC (L-ascorbic acid plus DHA) in the cerebrum, cerebellum, liver, kidney, soleus muscle, extensor digitorum longus (EDL) muscle, heart and plasma at 75 days after weaning. The total VC levels in all these tissues and plasma from VC(-) SMP30/GNL KO mice were <2% of the values obtained for the VC(+) SMP30/GNL KO mice (Fig. 3). Most of the latter values were similar to those of VC(+) and VC(-) WT mice. However, in the liver, kidney and plasma, total VC levels of VC(+) mice were approximately half of those in the VC(+) and VC(-) WT mice. The amount of DHA per total VC content in the cerebrum, cerebellum, liver, kidney, soleus muscle, EDL and heart was below 5% in three groups, VC(+) WT, VC(-) WT and VC(+) SMP30/GNL KO mice. In contrast, the percentage of DHA in plasma from VC(+) SMP30/GNL KO mice as well as VC(-) WT and VC(+) WT mice was 15.8%, 18.5% and 19.0%, respectively.

Total carnitine (acyl carnitine plus free carnitine) levels in the cerebrum, cerebellum, liver,

kidney, soleus muscle, EDL muscle, heart and plasma at 75 days after weaning are shown in Figure 4. No significant difference was noted in any of these tissues from VC(-) and VC(+) SMP30 KO mice. Moreover, the total carnitine levels of these two groups were not significantly different from those observed for VC(-) and VC(+) WT mice. However, the total carnitine level in the hearts from VC(-) KO mice was only 59.1% of that in the VC(+) KO mice; a similar decrease was observed in the VC(-) WT mice.

**Urinary carnitine excretion upon VC depletion.** Rebouche (34) reported that carnitine depletion in VC-deficient guinea pigs results from the decreased efficiency of carnitine reabsorption in kidney. To examine whether a VC deficiency influences urinary excretion of carnitine in SMP30/GNL KO mice, we measured the urinary carnitine from VC(-) SMP30/GNL KO mice and their VC(+) counterparts. At 30 and 80 days after weaning, both groups were evaluated for total carnitine levels in 24-h urine samples (Fig 5), but no significant difference was found. The urinary carnitine contents of VC(-) and VC(+) WT mice were also similar.

**In vitro carnitine biosynthesis assay.** For this assay, we used liver and kidney homogenates originating from VC(-) SMP30/GNL KO mice at 75 days after weaning. Liver homogenates contained endogenous enzymes for carnitine biosynthesis and had no detectable level of VC. When the liver homogenates were incubated at 37°C, the concentration of carnitine increased time-dependently for 60 min and then gradually decreased (Fig. 6A). Addition of 1 mM VC did not affect the course of that carnitine formation. On the other hand, the carnitine levels in kidney homogenates were not changed until 90 min of culture even when VC was added (Fig. 6B). To validate this assay system, we heated liver homogenates at 95°C for 5 min and then performed the carnitine biosynthesis assay. However, the carnitine levels did not change at all during incubation for 120 min (Fig. 6C).

**Tissue GSH levels after VC depletion.** GSH levels were then compared in the liver, soleus muscle, heart and serum from all four groups of test mice at 75 days after weaning. In livers from