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 ± 1.7 g and 23.9 ± 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 ± 0.3 ml/day/mouse for VC(+) KO mice and 3.2 ± 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

VC(-) SMP30/GNL KO mice, amounts of GSH were increased by 44.5%, 32.8% and 30.8%, respectively, above levels in livers from VC(+) SMP30/GNL KO, VC(+) WT and VC(-) WT mice, but the differences among the four groups were not statistically significant (Fig. 7). On the other hand, GSH levels in the soleus muscles and plasma from VC(-) SMP30/GNL KO mice were 16.4% and 45.5%, respectively, lower than in VC(+) SMP30/GNL KO mice, although the GSH content in heart tissues from VC(-) SMP30/GNL KO mice were not changed when compared to the other three groups.

DISCUSSION

Despite the traditional belief that VC is an essential cofactor for the activation of γ-BBD and TMLD in the carnitine biosynthesis pathway *in vivo*, results from the present study using VC-depleted SMP30/GNL KO mice challenge this assumption. Our conclusion to the contrary stems from experiments presented here in which these VC(-) SMP30/GNL KO mice successfully produced carnitine despite the animals' VC insufficiency.

SMP30 is an age-associated protein that decreases with aging in the liver, kidney and lungs in an androgen-independent manner (41-43). SMP30 was first discovered in 1992 by using a proteomic analysis that compared soluble proteins from the livers of young and old rats (41). Although the decreased expression of SMP30 during aging was immediately apparent, the physiological functions of SMP30 remained obscure for more than ten years (44). To resolve this issue, we developed SMP30 KO mice by gene targeting (36). During subsequent experiments performed *in vivo* and *in vitro*, SMP30 KO mice proved far more susceptible to TNF-α- and Fas-mediated apoptosis than their WT counterparts (36) and showed abnormal accumulations of lipids including triglycerides, cholesterol and phospholipids in the liver (45,46). Recently we found that SMP30 is a GNL involved in the VC biosynthetic pathway. Further study showed that SMP30/GNL KO mice could not synthesize VC *in vivo* and that they developed symptoms of scurvy when fed a VC-deficient diet (35).

The SMP30/GNL KO mice used here were fed a VC- and carnitine-deficient diet (Table 1) and water containing no VC for 75 days after weaning, which began when the animals were 40-days-old. Initially, these VC(-) SMP30/GNL KO mice grew as well as matching VC(+) mice as evident from their increased body weight for the first 40 days followed by a gradual decrease (Fig. 2). After another 30 days, the VC(-) KO mice weighed 32% less than the VC(+) controls given supplementary VC. Additionally, multiple tissues and plasma of the SMP30/GNL KO mice had less than 2% the total VC content of VC(+) SMP30/GNL KO, VC(+) WT and VC(-) WT mice (Fig. 3). However, total carnitine levels in various tissues, except the heart, unexpectedly showed no difference among these four groups (Fig. 4). The abundance of carnitine in the heart and skeletal muscle has been attributed to these tissues' production of energy via β-oxidation from long-chain fatty acids for muscle expansion (7). In the present study, total carnitine levels in hearts from VC(-) SMP30/GNL KO and VC(-) WT mice were about half the amounts in VC(+) SMP30/GNL KO and VC(+) WT mice. Still unclear is whether VC(+) groups generated an increased quantity of carnitine or the VC(-) group underwent a decrease of carnitine; however, this phenomenon may be related to the requirement for extra carnitine storage in the heart for use in energy production.

In mammals, carnitine homeostasis is maintained by adsorption from dietary sources, endogenous synthesis and efficient tubular reabsorption by the kidney. *In vivo*, VC depletion in tissues and plasma may be a manifestation of the inhibitory effects of carnitine excretion from tissues and an increase in the efficiency of tubular reabsorption from the kidney. To examine the effects of VC depletion and carnitine deficiency on the urinary excretion of carnitine, we measured total carnitine levels in urine collected for 24 h periods at 30 and 80 days after feeding of a VC- and carnitine-deficient diet. However, the total carnitine levels in urine were quite similar in all four groups of test mice at those time intervals (Fig. 5). These results denote that the VC deficiency in tissues and plasma had no influence on the urinary carnitine excretion of SMP30/GNL KO mice.

However, many reports indicate that tissue carnitine levels are significantly decreased in VC-depleted guinea pigs *in vivo* (30-32). Alkonyi *et al.* (33) reported that an increase of urinary excretion greatly contributed to the loss of carnitine in

guinea pigs during states of VC deficiency and starvation. Rebouche (34) also stated that carnitine depletion in VC-deficient guinea pigs resulted from a decreased efficiency of carnitine reabsorption in their kidneys. These reports strongly assert that subnormal levels of VC in various tissues of VC-depleted guinea pigs were caused by a rise in urinary carnitine excretion. If so, guinea pigs cannot be used to determine the involvement of VC in carnitine biosynthesis. In the present study the amount of carnitine excreted in the urine of SMP30/GNL KO mice was no different in samples from VC-depleted *versus* VC-supplemented animals (Fig. 5). Although the difference between guinea pigs and SMP30/GNL KO mice is still unclear, SMP30/GNL KO mice are a far more suitable animal model for determining the necessity of VC in carnitine biosynthesis.

We further examined carnitine biosynthesis by using VC-depleted liver and kidney tissues from SMP30/GNL KO mice. Humans, cats, cows, hamsters and rabbits can synthesize carnitine in the liver and kidney because they have γ-BBD activity in those tissues; however, mice, rats, sheep, dogs and guinea pigs synthesize carnitine only in the liver, because their kidneys lack γ-BBD activity entirely or contain only very low levels (22-24). In the present study of liver homogenates from VC-depleted SMP30/GNL KO mice with and without 1 mM VC, there was no difference in carnitine production (Fig. 6A), In addition, no carnitine production was detectable in the kidney samples according to the same assay system (Fig. 6B). These results strongly suggest that VC is not essential for carnitine biosynthesis *in vitro*. Vlies *et al.* (24) reported that both TMLD and γ-BBD activities were reduced when VC was removed from their complete assay mixture *in vitro*. However, Punekar *et al.* (29) observed a small amount of carnitine synthesis, but not VC, in the presence of GSH, in their assay mixture. Further, a combination of GSH and glutathione peroxidase yielded a large amount of carnitine synthesis, suggesting that GSH may effectively replace VC in the carnitine biosynthetic pathway. In fact, in the livers from our VC(-) SMP30/GNL KO mice, GSH levels were higher by 31 to 45% than in those from the other three groups tested, although the difference was not statistically significant (Fig. 7). Conversely, GSH levels in soleus muscles and plasma of VC(-) SMP30/GNL KO mice were lower than that from the other three groups. These results reinforce the likelihood that GSH may replace VC in the carnitine biosynthetic pathway *in vivo* when VC is depleted in liver.

In conclusion, our results indicate that VC is not essential for carnitine biosynthesis *in vivo*, as verified by the clearcut presence of carnitine in VC-depleted SMP30/GNL KO mice. Additionally, GSH may compensate for VC in the event of the latter's depletion. Finally, our model of SMP30/GNL KO mice provides an optimal opportunity for investigating the necessity of VC in carnitine biosynthesis.

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FOOTNOTES

*This study is supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan (to A.I., S.H. and N.M.), and a Grant-in-Aid for Smoking Research Foundation, Japan (to A.I.) and the Suzuken Memorial Foundation, Japan (to A.I.). We thank Ms. P. Minick for the excellent English editorial assistance. Vitamin C powder was kindly provided by DSM Nutrition Japan.

The abbreviations used are: γ-BB, γ-butyrobetaine; γ-BBD, γ-BB dioxygenase; DHA, dehydroascorbic acid; DTT, dithiothreitol; EDL, extensor digitorum longus muscle; GNL, gluconolactonase; GSH, reduced glutathione; HPLC, high-performance liquid chromatography; KO, knockout; PCA, perchloric acid; SMP30, senescence marker protein-30; TMABA, 4-N-trimethylaminobutyraldehyde; TML, 6-N-trimethyllysine; TMLD, TML dioxygenase; HTML, 3-hydroxy-6-N-trimethyllysine; VC, vitamin C; WT, wild type

FIGURE LEGENDS

Fig. 1. The pathway of carnitine biosynthesis with proposed involvement of VC. L-lysine residues in proteins are trimethylated by specific methyltransferases that use S-adenosyl-L-methionine as the methyl donor to TML. After its release by protein degradation, TML is hydroxylated by TMLD to HTML. HTML is cleaved by 3-hydroxy-6-N-trimethyllysine aldolase to TMABA and glycine. TMABA is oxidized to γ-BB by 4-N-trimethylaminobutyraldehyde dehydrogenase. In the last step, γ-BB is hydroxylated to L-carnitine by γ-BBD. In the present study, we found VC is not an essential cofactor for the activation of γ-BBD and TMLD in the carnitine biosynthesis pathway.

Fig. 2. Body weight changes of VC(+) and VC(-) groups composed of WT and SMP30/GNL KO mice. After the mice were weaned at 40 days of age (indicated at day 0), their body weights were measured for 75 days, and the mean body weight changes (difference from the mean body weight at day 0) were plotted. The final body weights of VC(+) SMP30/GNL KO, VC(-) SMP30/GNL KO, VC(+) WT and VC(-) WT mice at day 70 were 35.2 ± 1.7 g, 23.9 ± 1.9 g, 33.9 ± 0.5 g and 39.1 ± 0.5 g, respectively. Values are expressed as means ± SEM of five animals.

Fig. 3. Total VC levels in the cerebrum, cerebellum, liver, kidney, soleus muscle, extensor digitorum longus muscle (EDL), heart and plasma from VC(+) and VC(-) groups from WT and SMP30/GNL KO mice. Mice were supplied with or deprived of VC in drinking water for 75 days, starting when they were weaned at 40 days of age. Bars represent DHA (pink column) and ascorbic acid (green column). Values of total VC (DHA plus ascorbic acid) are expressed as means ± SEM of five animals.

Fig. 4. Total carnitine levels in the cerebrum, cerebellum, liver, kidney, soleus muscle, extensor digitorum longus muscle (EDL), heart and serum from VC(+) and VC(-) groups of WT and SMP30/GNL KO mice. Mice were supplied with or deprived of VC in drinking water for 75 days, starting when they were weaned at 40 days of age. Values of total carnitine are expressed as means ± SEM of five animals.

Fig. 5. Excretion of total carnitine in the urine at (A) 30 days and (B) 80 days after weaning of VC(+) and VC(-) groups of WT and SMP30/GNL KO mice. Each mouse was housed in a metabolic cage, and urine was collected for 24 h. Values of total carnitine were normalized by creatinin values and expressed as means ± SEM of five animals.

Fig. 6. *In vitro* carnitine biosynthesis assay. VC-depleted liver and kidney homogenates were prepared as described in "Experimental Procedures." For the carnitine biosynthesis assay, homogenates were incubated with 1 mM VC (pink circle) or without VC (green square) at 37°C for the indicated times. (A) Total carnitine concentrations in liver homogenates during incubation. (B) Total carnitine concentrations in kidney homogenates during incubation. (C) Total carnitine concentrations in VC-depleted liver homogenates heated at 95°C for 5 min then incubated at 37°C for 60 and 120 min. Values are expressed as means ± SEM of four samples.

Fig. 7. GSH levels in the liver, kidney, soleus muscle, heart and plasma from VC(+) and VC(-) groups from WT and SMP30/GNL KO mice. Mice were supplied with or deprived of VC in drinking water for 75

days, starting when they were weaned at 40 days of age. Values of GSH are expressed as means ± SEM of five animals.

Table I
Diet composition of CLEA-purified diet

Nutritional component (in 100 g)	
Moisture (g)	8.0
Crude protein (g)	20.4
Crude fat (g)	6.0
Crude fiber	3.0
Crude ash (g)	6.2
Nitrogen-free extract (NFE) (g)	56.4
Calorie (kcal)	361.2

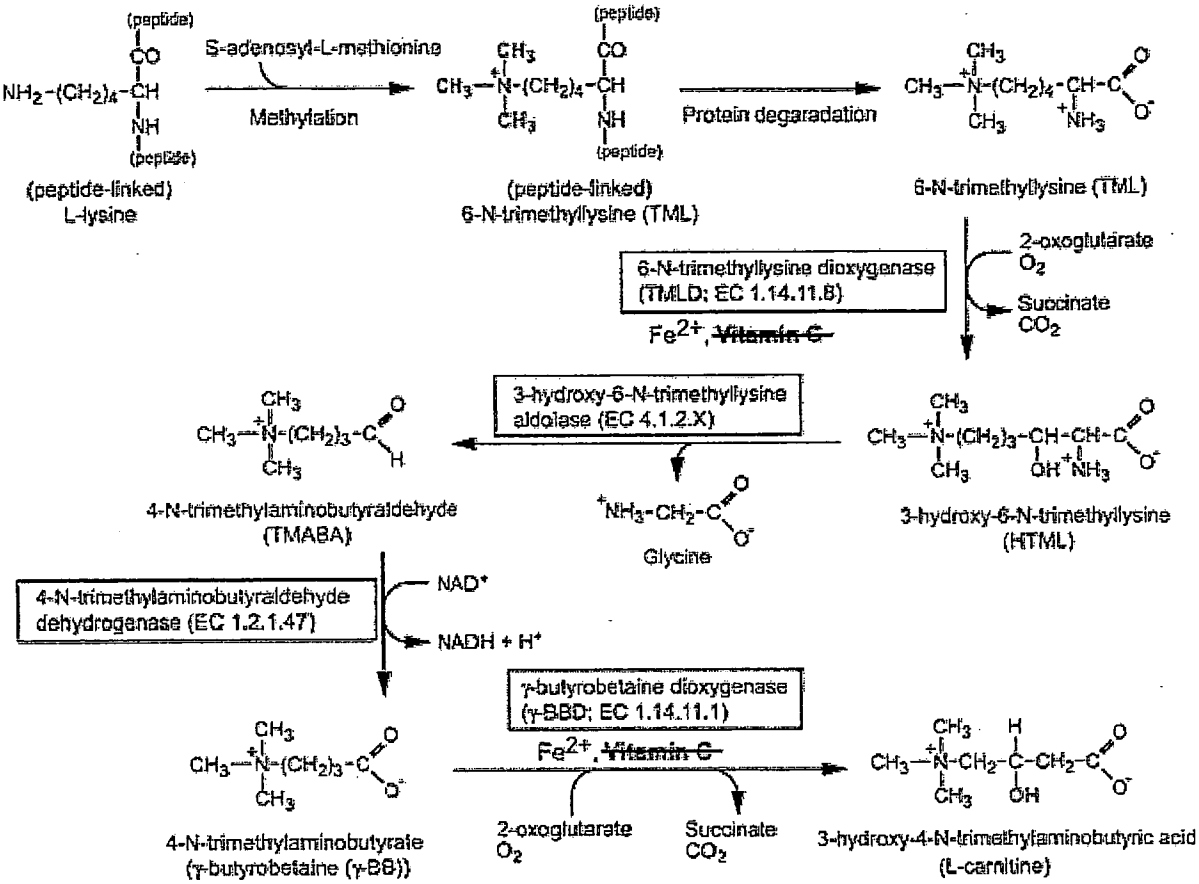
Minerals (in 100 g)	
Ca (g)	0.89
P (g)	0.66
Mg (g)	0.08
Na (g)	0.23
K (g)	0.50
Fe (mg)	31.70
Cu (mg)	0.32
Zn (mg)	3.46
Co (mg)	0.10
Mn (mg)	3.51
Ca/P	1.35
Ca/Mg	11.13
K/Na	2.17

Vitamins (in 100 g)	
Vitamin A (mg)	1.65
Vitamin D_3 (μg)	25
Vitamin E (mg)	20
Vitamin K_3 (mg)	0.3
Vitamin B_1 (mg)	1.5
Vitamin B_2 (mg)	1.6
Total vitamin C (mg)*	ND
Vitamin B_6 (mg)	1.0
Vitamin B_{12} (μg)	0.5
Pantothenic acid (mg)	4.0
Niacin (Nicotinic acid) (mg)	10.2
Folic acid (mg)	0.2
Choline (mg)	300
Biotin (μg)	500
Inositol (mg)	15

Other component (in 100 g)	
Total carnitine (mg)*	ND

ND, not detectable.
Data from CLEA Japan, Tokyo, Japan.
*Total VC and total carnitine were measured as described in "Experimental Procedures."

Figure 1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NH
(peptide)
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S-adenosyl-L-methionine
Methylation
CH3
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CH3
(peptide)
CO
NH
(peptide)
(peptide-linked) 6-N-trimethyllysine (TML)
Protein degaradation
CH3
CH3-N+-(CH2)4-CH-C
CH3
+NH3
O
O-
6-N-trimethyllysine (TML)
6-N-trimethyllysine dioxygenase (TMLD; EC 1.14.11.8)
Fe2+, Vitamin C
2-oxoglutarate
O2
Succinate
CO2
3-hydroxy-6-N-trimethyllysine aldolase (EC 4.1.2.X)
CH3
CH3-N+-(CH2)3-C
CH3
H
4-N-trimethylaminobutyraldehyde (TMABA)
+NH3-CH2-C
O
O-
Glycine
CH3
CH3-N+-(CH2)3-CH-CH-C
CH3
OH
+NH3
3-hydroxy-6-N-trimethyllysine (HTML)
4-N-trimethylaminobutyraldehyde dehydrogenase (EC 1.2.1.47)
NAD+
NADH + H+
γ-butyrobetaine dioxygenase (γ-BBD; EC 1.14.11.1)
Fe2+, Vitamin C
CH3
CH3-N+-(CH2)3-C
CH3
O
O-
4-N-trimethylaminobutyrate (γ-butyrobetaine (γ-BB))
2-oxoglutarate
O2
Succinate
CO2
CH3
CH3-N+-CH2-C-CH2-C
CH3
H
OH
O
O-
3-hydroxy-4-N-trimethylaminobutyric acid (L-carnitine)

Figure 2

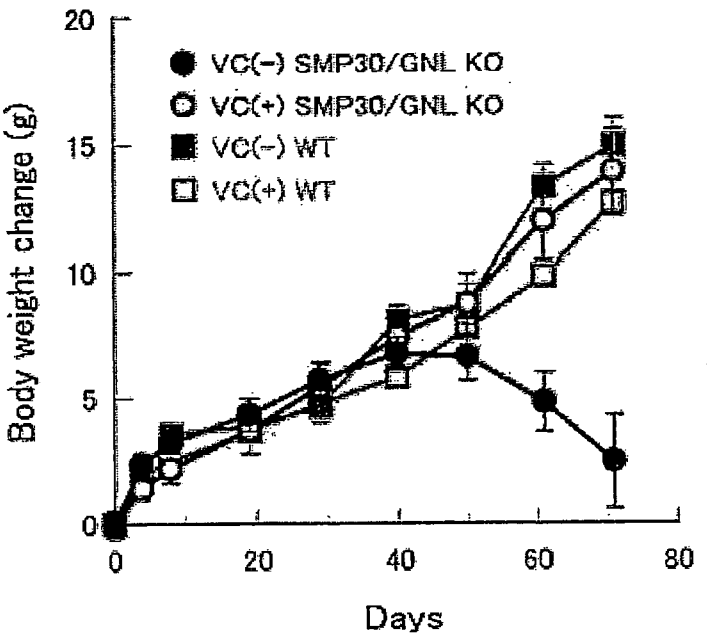

VC(−) SMP30/GNL KO
VC(+) SMP30/GNL KO
VC(−) WT
VC(+) WT
Body weight change (g)
20
15
10
5
0
0
20
40
60
80
Days

Figure 3

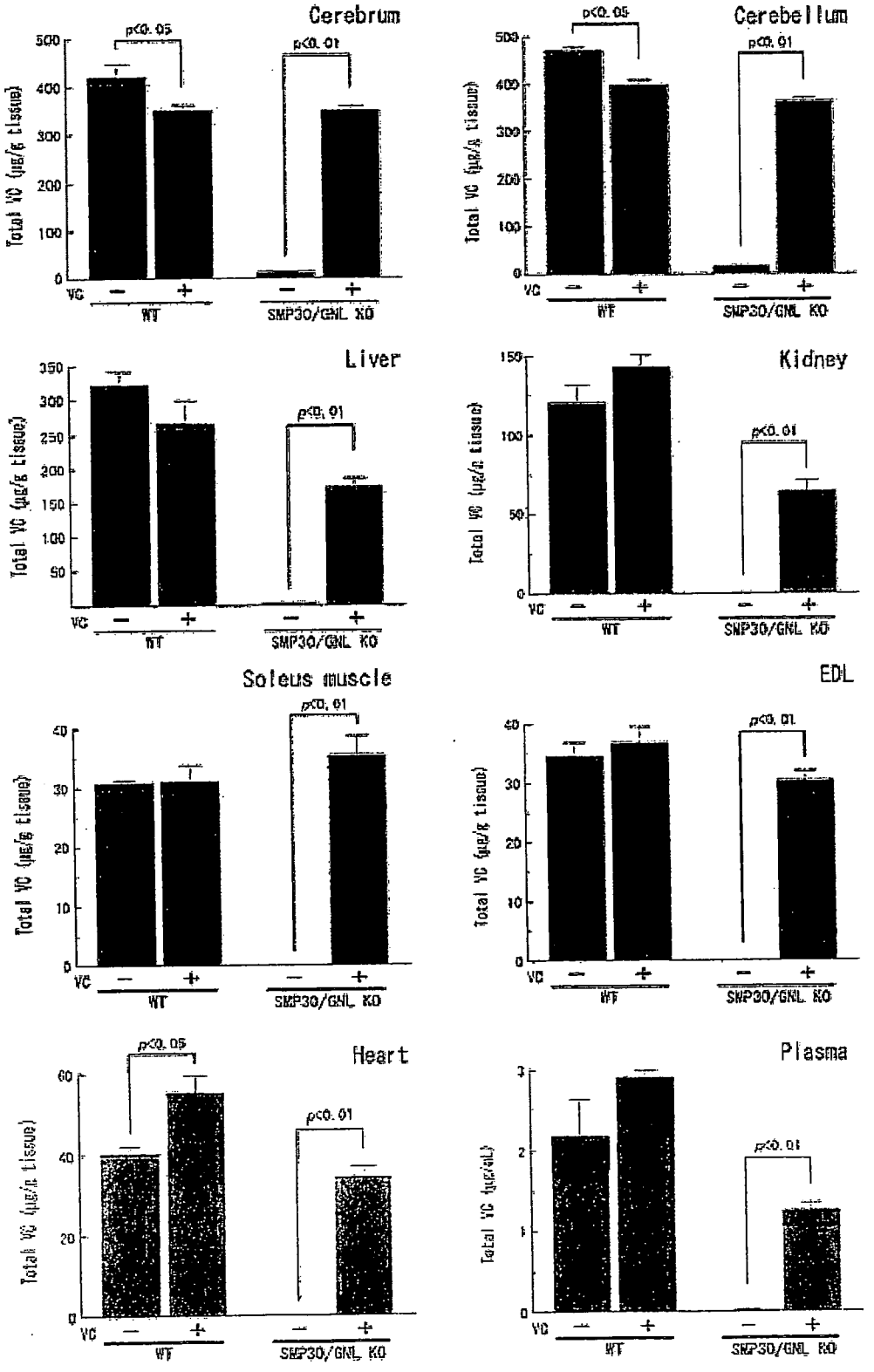

Cerebrum
Cerebellum
Liver
Kidney
Soleus muscle
EDL
Heart
Plasma
p<0.05
p<0.01
Total VC (μg/g tissue)
Total VC (μg/ml)
VC
WT
SMP30/GNL KO

Figure 4

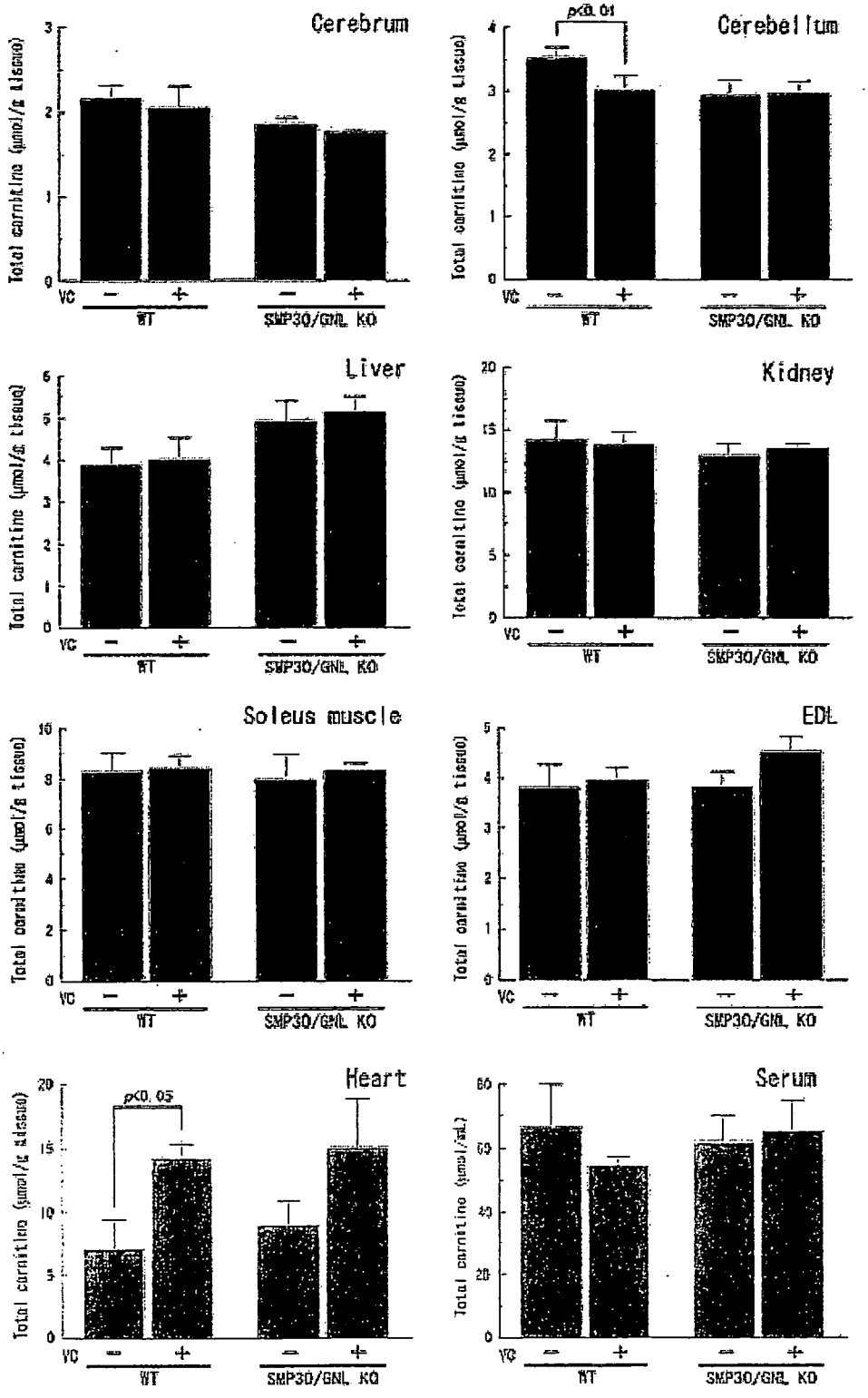

Cerebrum
Cerebellum
p<0.01
Liver
Kidney
Soleus muscle
EDL
Heart
p<0.05
Serum
Total carnitine (μmol/g tissue)
Total carnitine (μmol/mL)
VC
WT
SMP30/GNL KO

Figure 5

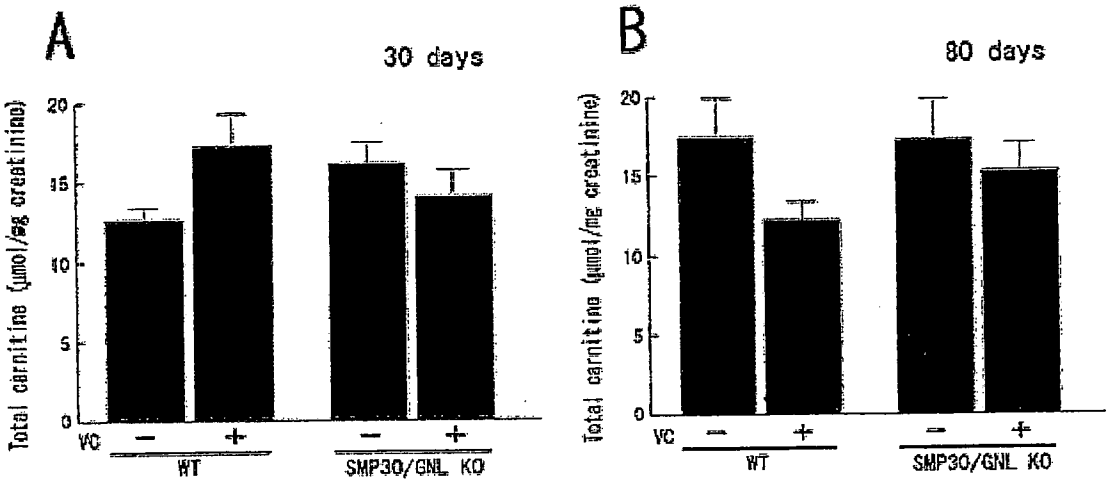

A
30 days
Total carnitine (μmol/mg creatinine)
20
15
10
5
0
VC
−
+
−
+
WT
SMP30/GNL KO
B
80 days
Total carnitine (μmol/mg creatinine)
20
15
10
5
0
VC
−
+
−
+
WT
SMP30/GNL KO

Figure 6

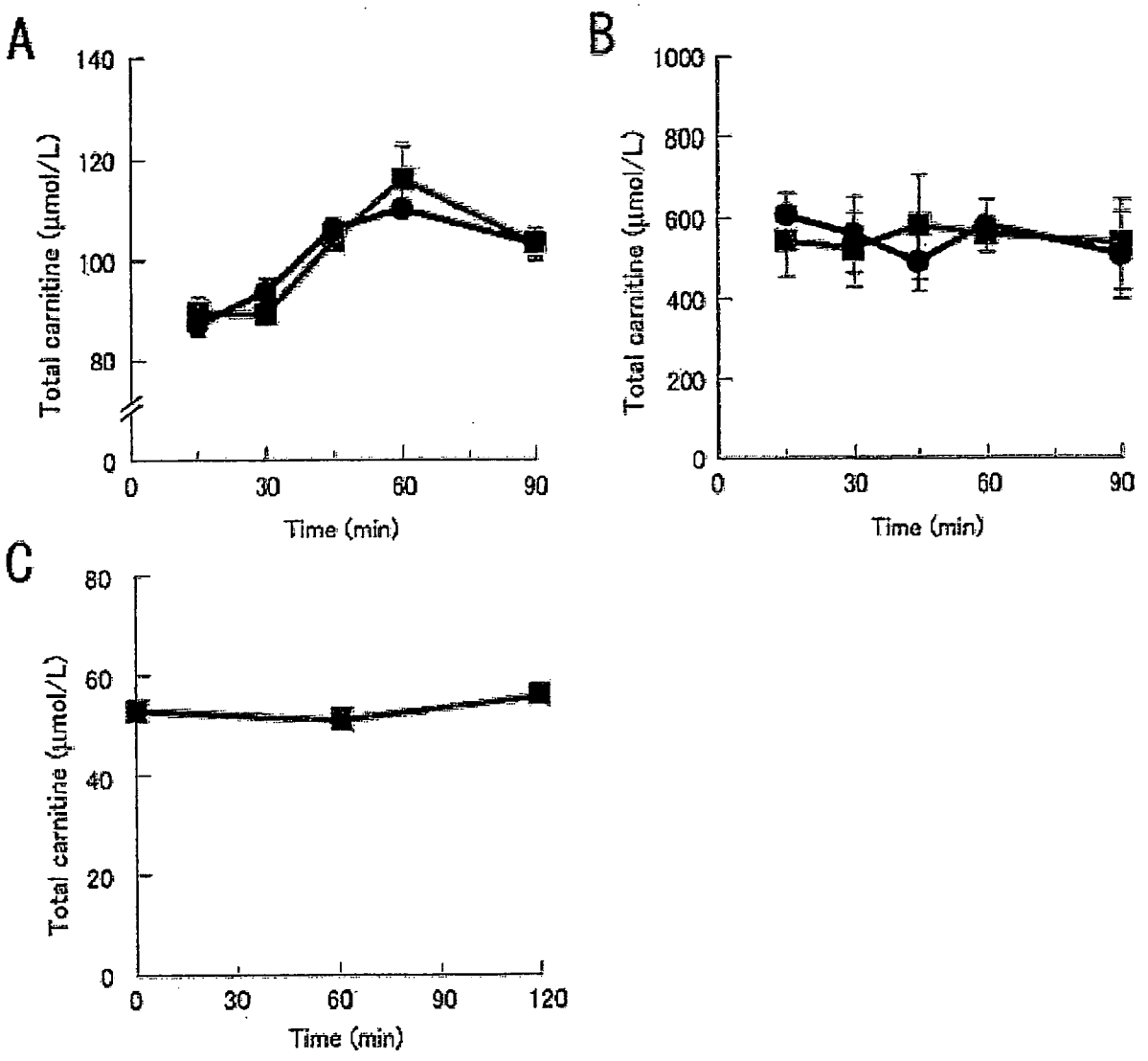
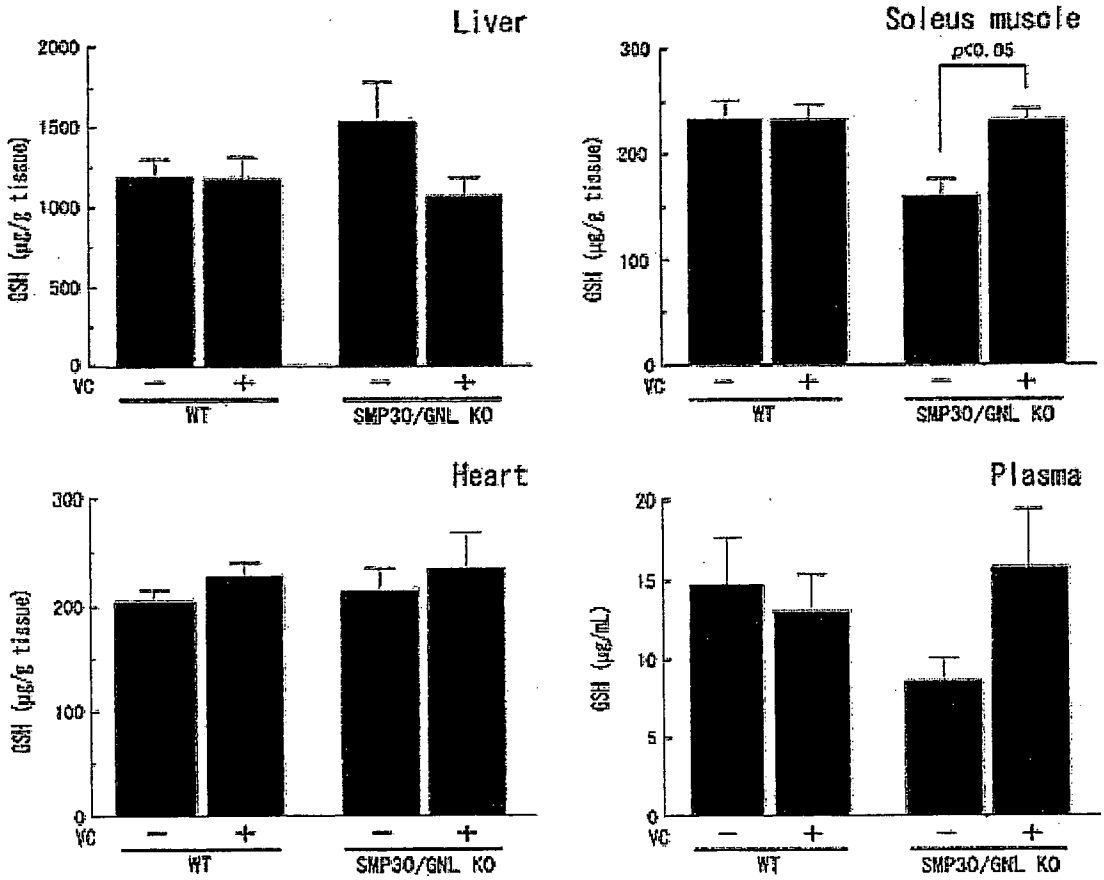

A
140
120
100
80
0
Total carnitine (μmol/L)
0
30
60
90
Time (min)
B
1000
800
600
400
200
0
Total carnitine (μmol/L)
0
30
60
90
Time (min)
C
80
60
40
20
0
Total carnitine (μmol/L)
0
30
60
90
120
Time (min)

Figure 7


Liver
GSH (μg/g tissue)
2000
1500
1000
500
0
VC
−
+
−
+
WT
SMP30/GNL KO
Soleus muscle
p<0.05
GSH (μg/g tissue)
300
200
100
0
VC
−
+
−
+
WT
SMP30/GNL KO
Heart
GSH (μg/g tissue)
300
200
100
0
VC
−
+
−
+
WT
SMP30/GNL KO
Plasma
GSH (μg/mL)
20
15
10
5
0
VC
−
+
−
+
WT
SMP30/GNL KO