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Morphometric Evidence of the Trophic Effect of *L*-Carnitine on Human Skeletal Muscle

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Abstract. We investigated the effect of long-term i.v. administration of *L*-carnitine on human muscle fibers using morphometric parameters. We administered 2 g/day *L*-carnitine to patients undergoing hemodialysis for at least 12 months. At the end of this period a marked increase in serum and muscle carnitine levels was observed in all patients, together with hypertrophy and predominance of type 1 fibers. *L*-carnitine was withheld for 4 months, during which time serum and muscle levels gradually decreased and no changes were observed in muscle fibers. Subsequent addition of *L*-carnitine to dialysis fluid for another 4 months stabilized lower levels. At the end of this period reduction of diameter of type 1 fibers was observed. Type 2 fibers remained unchanged. Moreover, type 1 fibers remained predominant in all cases. Hence, we suggest that carnitine has a specific trophic effect on type 1 fibers which are characterized by an oxidative metabolism.

Introduction

L-carnitine is known to transfer long-chain fatty acids from the cytoplasm across the inner mitochondrial membrane [1–8]. It is an essential metabolite in those organs and tissues such as the myocardium and skeletal muscle which preferentially use fatty acids for their energy requirements. To date we have only a limited and indirect knowledge of the effect of carnitine on human muscle tissue, obtained from studies on subjects with a genetic deficiency of this metabolite [9–12]. Investigations into the role of carnitine in hemodialysis have been prompted by the fact that chronic loss of this metabolite during dialysis [13–19] has been related to complications which often accompany intermittent hemodialysis, such as congestive cardiomyopathy and the postdialysis syndrome, characterized by a progressive proximal limb myopathy with muscle weakness, asthenia and cramps. Exogenous *L*-carnitine administration has been shown to improve muscle symptoms [15–22]. Thus, patients undergoing chronic hemodialysis constitute an efficient experimental model since endogenous carnitine can be easily replaced by the exogenous metabolite. The specific aim of this study was to determine whether or not prolonged increase in serum and muscle carnitine levels is related to modifications of the enzymatic pattern of muscle and the morphology of single fibers.

Materials and Methods

Experimental Model

Twenty-two patients aged 66 ± 12 years (12 males and 10 females), affected with chronic uremia and submitted to hemodialysis thrice weekly (each dialysis 3 h) for a period of 70 ± 59 months (1969 to 1984) were admitted to the study (fig. 1). Written informed patient consent had been previously obtained. Two grams *L*-carnitine were administered i.v. immediately after each dialysis for at least 12 months (mean 33 ± 13 months) as treatment for post-dialysis symptoms (hypertriglyceridemia, muscle weakness and cramps) present in varying degrees in all patients. Tolerance was excellent and no side effects arose. Clinical and laboratory data on these same patients have been published elsewhere [23]. In brief, hypertriglyceridemia, muscular weakness and cramps experienced prior to *L*-carnitine treatment subsequently disappeared in all patients. At the end of treatment, deltoid muscle biopsy was performed for histopathological studies and determination of muscle carnitine levels and morphometric analysis. Four months after the end of treatment a second muscle biopsy was performed as a control. For

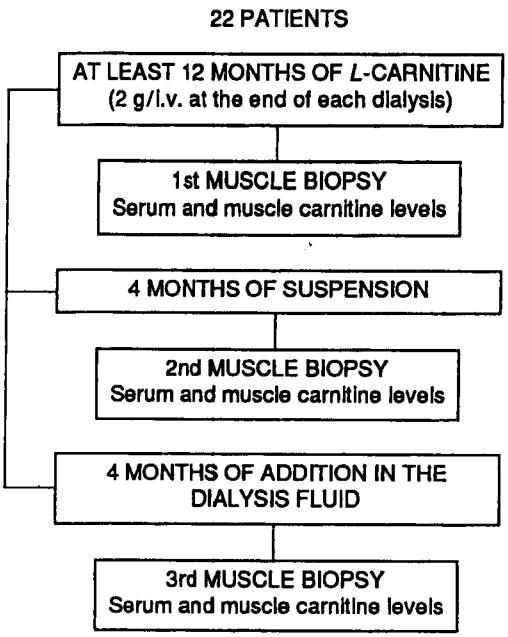

Fig. 1. Study design.

the following 4 months carnitine was added to the dialysis fluid in order to stabilize serum and muscle levels at normal values. At the end of this time a third biopsy was taken from the deltoid muscle. Samples for carnitine assay were immediately frozen in liquid nitrogen at -70 °C. Samples for histological studies were frozen in isopentane cooled in liquid nitrogen, according to the method of Dubowitz [24]. Serum carnitine was assayed at the time of biopsy.

Selection Criteria

Patients. Our patient population was homogeneous as regards both the clinical profile and the cause of renal insufficiency which in all cases was chronic glomerulonephritis. All patients were in apparently good clinical condition considering their pathology, were able to lead an independent life and had in common type of dialysis and equipment used, diet and absence of concomitant pathology (diabetes, etc.) likely to affect carnitine concentrations. Unimat (Belco) artificial kidneys with 11.5 μm Gambrio-Lundia filters were used and the patients received a daily diet comprising 1.2 g/kg proteins and 80-90 g lipids (2,300±300 total calories). All patients were treated with calcium carbonate as a phosphate binder.

Specimens. Bioptic specimens were to be free of artifacts due to transport or otherwise and constituted by at least 500 evaluable muscle fibers. All three biopsies for each patient were to be morphometrically and statistically comparable. Only 6 of the 22 patients enrolled in the study (4 females and 2 males aged between 56 and 72 years) exactly fitted these criteria. Nevertheless, morphometric data on muscle fibers were calculated for all biopsies free of artifacts. Artifacts due to freezing techniques could be recognized as holes in the fibers formed by ice crystals which caused the fibers themselves to swell. Moreover, faulty cutting techniques resulted in fields of fibers which were not in transverse section and could not be measured. The mean diameter of type I fibers in all 22 patients originally enrolled in the study (table I) did not differ significantly from that in the 6 patients selected for study (see table 3).

Table 1. Mean diameter of type I fibers in all 22 patients originally enrolled in the study (mean values ± SEM)

Patient	1st biopsy	2nd biopsy	3rd biopsy
1 P.E.	87.7±12.5	79.3±10.2	61.9±8.5
2 P.A.	67.5±11.8	78.5±10.3	58.9±9.9
3 B.E.	85.6±13.3	79.3±14.0	55.4±8.3
4 R.I.	65.4±10.9	71.6±12.7	47.1±11.1
5 R.I.	85.4±11.9	88.4±11.1	68.5±11.8
6 V.I.	75.4±11.8	79.1±8.1	76.8±11.7
7 P.A.	81.1±13.0	71.5±17.6	*
8 E.S.	67.7±18.2	63.0±17.9	*
9 B.L.	88.3±10.8	63.0±10.6	*
10 P.A.	66.0±10.5	*	*
11 O.D.	77.6±16.4	*	*
12 I.A.	74.9±12.6	70.5±13.8	*
13 M.A.	75.1±11.1	80.0±13.4	*
14 O.S.	82.6±10.1	*	53.0±8.9
15 G.A.	84.3±20.3	*	49.5±9.1
16 M.O.	*	75.1±16.7	*
17 F.R.	*	84.0±15.2	61.8±8.0
18 M.A.	*	*	45.0±13.8
19 P.I.	*	*	52.4±10.1
20 F.E.	*	77.2±11.1	*
21 P.E.	*	*	*
22 R.O.	*	*	*
Mean value	78.2±13.0	75.7±13.0	57.3±10.1
		$p<0.0002$	
		$p<0.0002$	

Asterisks denote bioptic specimens with marked artifacts.

Table 2. Muscle and serum *L*-Carnitine levels (mean values ± SEM)

Assay	1st biopsy	2nd biopsy	3rd biopsy
Total carnitine			
Muscle μmol/g NCP	51.9±11.7	25.2±5.8	19.2±3.9
		$p<0.01$	
		$p<0.01$	
Serum, μmol/l	1,297.1±255.70	101.2±11.5	121.1±18.2
		$p<0.01$	
		$p<0.01$	
Free carnitine			
Muscle	49.5±16.2	24.1±6.4	20.1±3.7
		$p<0.005$	
		$p<0.005$	
Serum	756.2±335.7	63.3±18.3	76.2±22.5
		$p<0.005$	
		$p<0.005$	

Serum Chemistry and Hematology

The following parameters were monitored on a routine basis in serum and whole blood: lipids (triglycerides, cholesterol, HDL-cholesterol, apoprotein A), hemoglobin, hematocrit, red and white blood cell counts, platelets, albumin, alkaline phosphatase, bilirubin, calcium, chloride, creatine phosphokinase, creatinine, globulin, glucose, phosphorus, potassium, serum glutamic-oxalacetic transaminase, serum glutamic-pyruvic transaminase, sodium, total protein, urea nitrogen and uric acid. Timing of collection and technical procedures to obtain serum were as previously described [23]. Moreover, serum aluminum assays were performed every 6 months in all patients.

Muscle and Serum Carnitine Assay

Muscle Carnitine

Muscle biopsies from the belly of the deltoid (open biopsy) were obtained at baseline, following a 4-month wash-out period and at the end of the study. The muscle specimens were immediately frozen at –70 °C. The tissue fragments (weight 20 mg on removal) were homogenized with 4 ml of cold methanol in a glass Potter homogenizer placed in an ice bath. The homogenate was then centrifuged for 10 min at 0 °C at 7,000 rpm. The supernatant, separated from the protein pellet by decanting and divided into two glass tubes, was dried by an air-stream. The content of one tube dissolved in distilled water was utilized for the determination of free carnitine. Two milliliters of chloroform were added to remove the lipids. The second tube was used for the determination of total carnitine. After further centrifuging at 7,000 rpm the free carnitine in the supernatant was determined by the method of Pearson et al. [25] with automatic equipment (Kinetic Analyzer 2086 Mark II, LKE).

Muscle Free-Carnitine Assay. To 100 µl of supernatant (containing free carnitine at concentrations between 1.5 and 15 µ*M*) 500 µl of reagent containing the following substances were added: Tris, DTNB, acetyl-CoA, EDTA and carnitine-acetyltransferase. The concentrations of these compounds in the assay mixture were: 70 m*M*, 0.07 µ*M*, 0.108 m*M*, 0.93 m*M* and 460 m*M*, respectively. Changes in optical density were observed at 410 nm. Concentrations were determined during the initial phase of the reaction which maintained linearity for 30 s after the sample was mixed with the reagents.

Free carnitine value is expressed in µ*M*/g of noncollagen protein (NCP) which was assayed in the residual protein pellet obtained after removing the methanol extract.

Noncollagenous Protein Assay. The pellet was hydrolyzed with 2 ml of NaOH (50 m*M*) at room temperature for 18 h. After centrifuging, 0.1 ml of supernatant was mixed with 0.6 ml of biurent reagent and then maintained at room temperature for 30 min. Noncollagenous protein concentrations were determined by reading optical density at 546 nm.

Muscle Total Carnitine Assay. The residue of the second tube was dissolved in 0.25% Triton X-100 saponified at pH 12 at 37 °C and neutralized with HCl for total carnitine determination as described for free carnitine assay.

Serum Carnitine

Predialysis blood samples were obtained following overnight fast and prior to heparinization at baseline, following a 4-month wash-out period, and at the end of the study. Total and free carnitine were determined in serum samples by the same methods described for the muscle concentrations. For free carnitine determination, the proteins were first removed by the ultrafiltration method (Amicon membrane cones) [26]. Under the above conditions, the recovery of free carnitine was in excess of 90% in both serum and muscle.

Morphometric Analysis

Morphometric analysis was performed using a Quantimet 920 image analyzer (Cambridge Instruments) on sections stained with ATPase preincubated at pH 4.2 and 9.3. For each biopsy, at least 500 fibers were studied.

The following parameters were evaluated.

(a) Mean diameter of muscle fibers:

$$D = 2\sqrt{A/\pi},$$

where A = area of fibers in transverse section. Fiber diameter was measured using the equivalent area diameter method [27, 28].

(b) Standard deviation (SD) of diameter.

(c) Coefficient of variability [24, 29]: (maximum diameter – minimum diameter)/SD × 100.

(d) Hypertrophy and atrophy factors, which indicate respectively the proportion of fibers larger than 80 µm in males (70 in females) and the proportion of fibers smaller than 40 µm in males (30 in females) [24, 29].

(e) Percent ratio type 1/type 2 fibers.

Statistical Analysis

The mean, median, SEM and range were calculated for each parameter. In order to evaluate the difference between the first, second and third muscle biopsies, the Wilcoxon nonparametric test for paired data and the Mann-Whitney U test were applied [30]. Moreover, the coefficient of correlation between carnitine levels and morphometric indices was calculated. Values of $p < 0.05$ were considered statistically significant.

Results

Clinical Parameters

Proximal muscular weakness and cramps did not reappear when *L*-carnitine therapy was withdrawn, which can most likely be related to the short period of withdrawal. Minor changes in serum chemical and hematological routine parameters were observed during the study that represent normal variations in the patient population. None of the observed differences were of clinical relevance. No side effects or adverse reactions were reported in any of the patients during the study.

Serum triglycerides, determined according to the method previously described [23], increased significantly from 190 ± 66 to 287 ± 151 mg/dl ($p < 0.01$) after the end of treatment with *L*-carnitine, but decreased again (no significant differences as compared with the baseline values) when carnitine loading was resumed.

Serum aluminum levels were consistently between 25 and 75 µg/l (below the level usually taken as indicating toxicity in dialysis patients, namely 100 µg/l [31]) in all patients.

Fig. 2. Patient 2: 1st biopsy, ATPase preincubated at pH 4.6. Predominance and hypertrophy of type 1 fibers (black) and reduction of number of type 2A fibers (white) can be observed, whereas type 2B fibers (gray) are normally present. × 78.

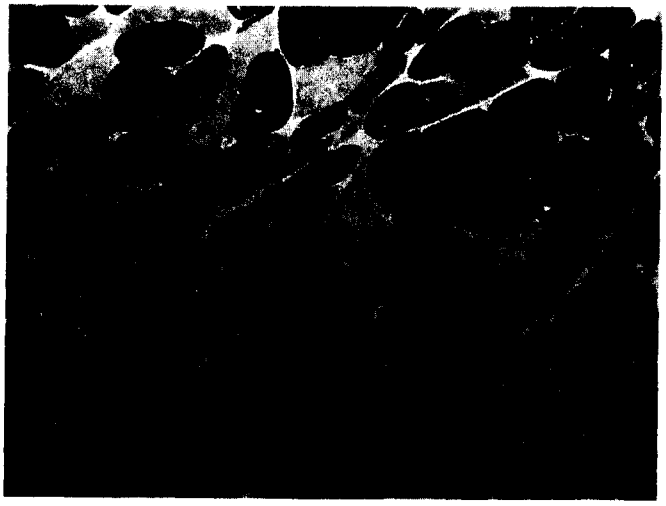

Fig. 3. Patient 2: 1st biopsy, ATPase preincubated at pH 4.6. Detail of figure 1. Most type 1 fibers (black) are hypertrophic and round in shape. Type 2B fibers (gray) are smaller than both type 1 and type 2A fibers (white). × 195.

Serum and Muscle Carnitine Levels (table 2)

After long-term administration, total carnitine muscle levels were 51.95 ± 11.73 μ*M*/g of NCP, in comparison to normal values (NV) of 20.3 ± 6.8 μ*M*/g [9], and serum levels were 1,297 ± 255.72 μ*M*/l (NV 150 ± 40 μ*M*/l). Free carnitine muscle levels were 49.48 ± 16.16 μ*M*/g of NCP (NV 17.6 ± 6.2) and serum levels were 756.16 ± 335.67 μ*M*/l (NV 44.1 ± 10). After treatment had been withheld, total muscle and serum carnitine levels decreased progressively and, after 4 months, reached values of 25.18 ± 5.82μ*M*/g of NCP and 101.18 ± 11.5 μ*M*/l, respectively. Likewise, free carnitine muscle and serum levels reached values of 20.08 ± 3.71 μ*M*/g of NCP and 76.16 ± 22.52 μ*M*/l, respectively.

The subsequent addition of carnitine to the dialysis fluid did not result in statistically significant differences as compared to these levels.

Histopathological Data

At the first biopsy, the only noteworthy finding observed in most of our patients was the focal presence of angulated or polygonal atrophic muscle fibers. These atropic fibers were type 2 and did not stain intensely with the NADH-diaphorase reaction. Type 1 fibers were round or polygonal in shape and most of them were hypertrophic, both in relation to the hypertrophy factor and as compared to the mean values reported in the literature for patients of the same age and for the same muscle [24, 29, 32, 33]. In all patients predominance of type 1 fibers and deficiency of type 2A fibers were observed (fig. 2, 3).

At the second biopsy no significant variation was observed in type 1 or type 2 fibers. At the third biopsy type 2 fibers showed similar characteristics to those of previous biopsies, whereas type 1 fibers seemed to have a smaller diameter (fig. 4). No other significant morphological alterations were observed and these fibers still predominated, whereas type 2A fibers had decreased in number.

Morphometry

The morphometric results are shown in tables 3 and 4. After long-term i.v. administration of *L*-carnitine, the mean diameter of type 1 fibers was 79.33 ± 3.48 μm (NV 60 ± 20 in men and 50 ± 20 in women [24, 29]). These fibers showed a marked hypertrophy, confirmed by a mean hypertrophy factor of 1,390.67 ± 282.55. At the first biopsy type 2 fibers had a mean diameter of 62.57 ± 4.34 μm (NV 60 ± 20 in men and 50 ± 20 in women [24, 29]). In only 1 case was an atrophy factor of type 2 fibers observed which was below normal values. Both types of fibers showed a normal variability. Moreover, type 1 fibers were predominant (64.21 ± 6.0%). At the second biopsy, neither type 1 nor type 2 fibers showed statistically significant variations in diameter or hypertrophy factor as compared to the first biopsy. Whereas in all patients the

Fig. 4. Patient 2: biopsy 3, ATPase preincubated at pH 4.2. Type 1 fibers (black) can be seen to be smaller than those observed at the first biopsy. Furthermore, most type 1 fibers are polygonal in shape. ×195.

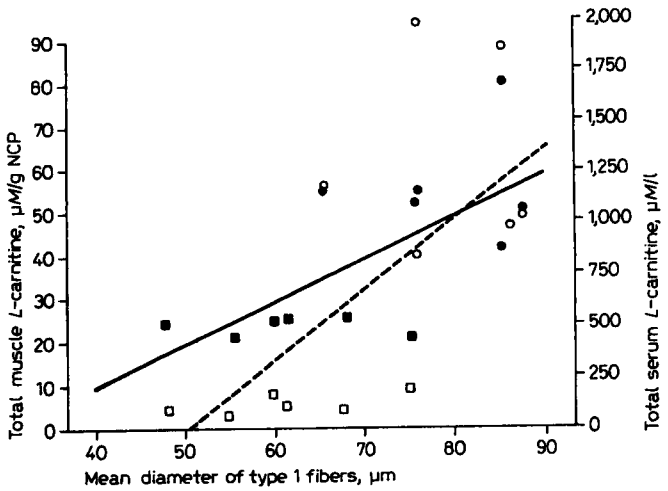

Fig. 5. Correlation between the mean diameter of type 1 fibers of first (circles) and third biopsies (squares) and corresponding total muscle (full circles and squares, full line) and serum (empty circles and squares, dashed line) *L*-carnitine levels. In both cases the coefficient of correlation was 0.64 ($p < 0.02$).

Table 3. Morphometric data of type 1 fibers (mean values ± SEM)

Parameters	1st biopsy	2nd biopsy	3rd biopsy
Mean diameter, μm	79.3 ± 3.5	79.4 ± 2.2 $p < 0.01$ $p < 0.02$	61.4 ± 4.2
SD of mean diameter	12.0 ± 0.3	11.1 ± 0.8	10.2 ± 0.6
Coefficient of variability	150.7 ± 4.2	130.3 ± 10.2	169.2 ± 14.3
Hypertrophy factor	1,390.7 ± 282.5	1,388.8 ± 226.3 $p < 0.01$ $p < 0.02$	384.5 ± 210.0
Atrophy factor	0	0	7.2 ± 4.9
Percent fibers	64.2 ± 6.0	56.6 ± 4.9	60.6 ± 3.8

Table 4. Morphometric data of type 2 fibers (mean values ± SEM)

Parameters	1st biopsy	2nd biopsy	3rd biopsy
Mean diameter	62.6 ± 4.3	60.8 ± 5.1	58.7 ± 8.4
SD of mean diameter	12.4 ± 1.5	12.0 ± 1.6	13.9 ± 2.3
Coefficient of variability	200.0 ± 28.1	202.0 ± 24.9	238.6 ± 17.1
Hypertrophy factor	317.2 ± 209.7	426.8 ± 119.6	710.8 ± 419.2
Atrophy factor	26.7 ± 26.7	78.0 ± 47.9	208.0 ± 156.7
Percent fibers	35.7 ± 6.0	43.4 ± 4.9	39.4 ± 3.8

atrophy factor and the coefficient of variability of type 1 fibers was within the normal range, in 3 patients type 2 fibers had a pathological atrophy factor and coefficient of variability. Type 1 fibers still predominated (50.59 ± 4.94%). Eight months after the end of treatment, at the third biopsy, the mean diameter to type 1 fibers was 61.43 ± 4.22 μm, whereas that of type 2 was 58.73 ± 8.37 μm. Type 1 fibers showed a statistically significant decrease in diameter, whereas no significant difference was observed in type 2 fibers when compared to the first and second biopsies. The coefficient of variability was normal in type 1 and increased in type 2 fibers. Type 1 fibers remained predominant. These fibers showed some hypertrophy, even though less than that observed for the first biopsy. In 2 patients the atrophy factor of type 2 fibers was extremely high (765 in patient 3 and 622 in patient 4).

Discussion

Whereas it is widely recognized that *L*-carnitine deficit is often associated with atrophy of type 1 fibers [9–12], to our knowledge this is the first time that a specific trophic effect of *L*-carnitine on type 1 fibers has been demonstrated. In previous studies [17, 34], we observed that despite carnitine deficiency, in hemodialysis patients typical morphological alterations usually seen in

muscle of congenital carnitine deficiency patients were absent. This seemed to be related to the fact that even though in these patients muscle carnitine falls by as much as 50%, levels as low as those found in congenital forms are never reached [13–19]. Histological changes in muscle would therefore seem to occur below a threshold value. Moreover, whereas short-time administration of carnitine causes the disappearance of muscle symptoms, there are no related morphological changes in muscle [17, 34].

In this study we have sought to correlate the increased serum and muscle levels resulting after long-term administration of carnitine with changes in the morphometric parameters of muscle fibers. Our study shows a direct correlation between carnitine levels and mean diameter of type 1 fibers when morphometric findings of the first and third biopsies are compared (fig. 5). In fact, there is a statistically significant ($p < 0.05$) reduction in the mean diameter and the coefficient of hypertrophy of these fibers of 22.6 and 72.3%, respectively (table 3). In apparent contrast with this downwards trend between the first and the third biopsies is the absence of changes in both morphometric parameters when the first and second biopsies are compared. It is to be noted that the latter was performed at the end of the period of gradual decrease in serum and muscle carnitine levels and not after exposure of the fibers themselves to constantly low levels of carnitine, which suggests that the muscle fibers adapt slowly to variations in carnitine levels and relative metabolic conditions.

The action of carnitine on type 1 fibers would seem to be selective. In our study, except for serum and muscle carnitine levels, there were no variations in clinical or laboratory parameters, nor was there any change in the morphometric parameters of type 2 fibers. Moreover, a direct effect of hemodialysis on the morphology of type 1 fibers can be excluded on the basis of the studies of Bautista et al. [22] and a previous study published by us [17]. In the latter we investigated 14 patients on intermittent hemodialysis over an average period of 23 ± 4 months (range 5–58 months) in the absence of substitution therapy (including carnitine). Muscle biopsies were used for light and electron microscopic studies. Morphologically, no pathological alterations were observed in either type 1 or type 2 muscle fibers of 13 patients. Light and electron microscopic studies of the muscle specimen of the 14th patient showed a typical nemaline myopathy with rod bodies in the cytoplasm most likely not related to dialysis.

Bautista et al. [22] reported 13 hemodialyzed patients who developed progressive paresis of the proximal muscles of the limbs. Biopsies of deltoid muscles of 10 of these patients showed selective atrophy of type 2 fibers probably due to osteomalacia caused by aluminum contamination in the dialysis fluid. None of our patients showed osteomalacia or encephalopathy due to aluminum intoxication, nor were the muscle symptoms those of proximal myopathy as described by Bautista et al. [22]. Moreover, in all patients aluminum levels were constantly within the range 25–75 μg/l (see Material and Methods). Furthermore, in none of the biopsies studied by us did type 2 fibers show pathological alterations, as occurs in muscle following osteomalacia from aluminum intoxication [22]. It remains to be seen whether or not the absence of a response by type 2 fibers is the expression of a normal behavior or of pathological alterations of these fibers themselves. In uremic patients, in fact, peripheral neuropathies have been reported in which changes of type 2 fibers and nonneurological atrophy involving exclusively this kind of fiber predominate [35, 36]. In our study it would seem possible to exclude this event on the basis of the mean variability coefficient and the mean atrophy factor of type 2 fibers. Both parameters, though high as compared to those of type 1 fibers, possibly because of focal atrophy observed only in 2 of our patients, remain within normal limits.

An explanation of the biochemical and molecular mechanism underlying the selective response of type 1 fibers does not fall within the scope of this study. However, it is known that type 1 fibers are characterized by an oxidative metabolism [24, 36–38] and that carnitine plays a key role in the transportation of fatty acids across the mitochondrial membrane [1–8, 39].

The pathogenetic mechanism of predominance of type 1 fibers, a common finding in neuromuscular pathology, is not clear, even if it seems to be in some way related to reinnervation or to a possible action linked to neurogenic stimulation [24, 36, 39]. In our patients, predominance of type 1 fibers occurred together with a strong reduction of type 2A fibers. It is known that the metabolic characteristic of type 2A fibers are intermediate between type 1 and type 2B fibers [9, 24, 36, 40]. The abnormal utilization of lipid metabolism due to an increased availability of carnitine would thus result in the modification of the enzyme pattern of these fibers. Hence, it seems reasonable to hypothesize a transformation of type 2A fibers into type 1. These were still predominant 8 months after the end of treatment, which suggests that this phenomenon is irreversible. Otherwise, it may be that this period is too short to permit a return to the original enzyme pattern of the muscle fibers.

For technical reasons, it was possible to perform complete morphometric examination of three biopsy specimens per patient in only 6 of the 22 patients enrolled. Nevertheless, despite the flaws in the study design and the limited number of patients we observed, our study would seem to show that in chronic hemodialysis patients undergoing long-term i.v. *L*-carnitine treatment (2 g/day), and thus having much higher serum and muscle levels than normal, there is marked hypertrophy and predominance of type 1 fibers. Further studies employing an ad hoc model including ultrastructural morphometry will be necessary in order to better clarify the morphogenesis of carnitine-induced hypertrophy.

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