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Docosahexaenoic acid (DHA) injection in spinal cord transection stimulates Na^+,K^+-ATPase in skeletal muscle via β 1 subunit

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Abstract
Spinal cord injuries (SCI) induce a loss of skeletal muscle mass and functional capacity. The muscle excitability and contractility depend on the plasma membrane potential, regulated by transmembrane ion gradients, and thus necessarily on the Na^+,K^+-ATPase activity. The aim of this work was to evaluate the consequences of a spinal cord transection (SCT) on the skeletal muscle Na^+,K^+-ATPase and the impact of collateral GlyceroPhosphoLipids enriched in DocosaHexaenoic Acid (GPL-DHA) administration. The Na^+,K^+-ATPase activity and membrane expression of Na^+,K^+-ATPase α1, α2 and β1 isoforms were assessed by K^+-stimulated para-nitrophenyl phosphatase (pNPPase) measurements and Western Blotting, respectively. The results show that spinal cord transection increased significantly ($p<0.05$) Na^+,K^+-ATPase activity in muscle by 25% and decreased the amounts of α1 isoform and α2 isoform expressions by 50% ($p<0.05$) respectively compared to controls. The results also show that early injection of GPL-DHA after SCT decreases in membrane skeletal muscle the α1 and α2 isoforms expression but increases the membrane Na^+,K^+-ATPase activity. This treament partially restores the membrane expression of the β1 subunit of the Na^+,K^+-ATPase. These data suggest that the increase of β1 subunit expression is probably the main trigger to the membrane Na^+,K^+-ATPase activation following a trans-synaptic denervation.

Key words: Na^+,K^+-ATPase, spinal cord transection, GPL-DHA, *biceps femoris* muscle, skeletal muscle, spinal cord injury, polyunsatuated fatty-acids.

Introduction

Spinal Cord Injury (SCI) is one of today's most important challenges for medical care and neurosciences (1). Resulting chronic disabilities, neuropsychological and social implications represent a heavy financial burden on the health care system. Therefore there is a major scientific interest to better understand mechanisms involved at the neural and muscular levels after a Central Nervous System (CNS) injury. For the patients, axonal regeneration and functional recovery are extremely limited. In addition, loss of skeletal muscle mass and functional capacity could have a dramatic impact on associated comorbidities (2-4), such as diabetes, obesity, lipid disorders, cardiovascular diseases, and mortality (5). These comorbidities are the consequences of the elevated incidence of muscle atrophy, deconditioning and the resulting physical inactivity.

The inflammatory reaction following spinal cord injury is one of the main acute mechanism involved, which could theoretically lead to eliminate pathogenic agents and clear debris. Inflammatory cells promote wound healing events that could support potential recovery. Nevertheless, this cascade of events in both the acute injured spinal cord period and during wound healing may be overshadowed by an excessive collateral accumulation of toxic molecules produced by inflammatory cells that damage otherwise intact tissue (6).

The central nervous system (CNS) is highly enriched in long chain polyunsaturated fatty acids (PUFA) from omega-3 series which have a structural role in all tissues (7) and an anti-inflammatory effect (8). It is well established that the incorporation of omega-3 fatty acids modifies inflammatory and immune reactions, making the omega-3 fatty acids potential therapeutic agents for inflammatory diseases. Their effects are brought by the modulation of the type and the amount of eicosanoids (9). Michael-Titus *et al.* (10) showed that the intravenous administration of Alpha-Linolenic Acid (ALA) or Docosahexaenoic Acid (DHA) 30 minutes after spinal cord hemi-transection in rats, have a significant neuroprotective effect. Indeed, it was observed a reduction of neuronal cell loss, oligodendrocytes loss, a decrease of apoptosis one week after injury, and an improve of the return of motor function (11). In another model of spinal cord compression (12), the injection of DHA 30 minutes after injury significantly reduced neuronal loss, increased survival of oligodendrocytes and increased one week after injury, the survival percentage of the anterior and posterior horn motor neurons. A decrease in the immune response to ED1 (marker of the inflammatory response) was also observed and improved the recovery of the locomotor function when the injury is followed by a post-traumatic diet enriched in DHA (13).

Recently, like Eicosapentaenoic acid (EPA) and DHA diets for months, an intravenous bolus injection of a fish oil triacylglycerol emulsion in human allows rapid enrichment in leukocyte and platelet phospholipids within 60 min and ≥ 24 hours after the injection (14). Two different molecular forms, triacylglycerol and phospholipids, of omega-3 polyunsaturated fatty acids affect the plasma level of EPA and DHA differently, with the most available for the phospholipid form (15). In this study, we tested the effect of an intravenous bolus injection with an emulsion of mainly DHA linked to phospholipids (16). This vector seems to be more resistant to oxidation with more cell membrane DHA accretion when compared to triacylglycerol or ethyl esters forms (17). This DHA form is a naturally present in poultry eggs. This glycerophospholipids enriched in docosahexaenoic acid (GPL-DHA) form was found to be neuroprotective on diabetic induced neuropathy (18).

In muscle, the excitability and contractility depend on the plasma membrane potential and the transmembrane ion gradients and thus necessarily on Na^+,K^+-ATPase activity. The electrogenic activity of the Na^+,K^+-ATPase is stimulated by a repetitive potential action and contributes to the maintenance of the sarcolemma's membrane potential and excitability (19). In humans, a decreased activity of Na^+,K^+-ATPase from muscular dystrophy has been observed (20), Moreover, in rats muscle inactivity appears to cause a decrease in the amount of Na^+,K^+-ATPase available in skeletal muscle (19) . We previously demonstrated that fish oil diet was efficient on the cardiac muscle (21) and during diabetes (22).

The aim of this work was to evaluate the effect of a spinal cord transection (SCT) and the impact of a collateral GPL-DHA injection on distal skeletal muscle Na^+,K^+-ATPase. To address this, we determined the K^+-dependent phosphatase activity of the Na^+,K^+-ATPase (K-pNPPase activity as sensitive to Na^+) and analyzed the immunological characterization of catalytic α (α1, α2) subunit and β1 subunit expression from crude membrane fractions of rat biceps femoris skeletal muscles.

Materials and methods

Animals

The study was conducted according to the guidelines of the French Ministry of Agriculture on the experimental use of laboratory animals with an agreement. The principles of laboratory animal care (NIH) were followed. A total of 24 adult female Wistar rats (220-240 g) were obtained from a Specific Pathogen Free (SPF) breeding colony (R. Janvier, Le Genest-St-Isle, France) and kept in standardized cages (type 4, Macrolon) on a 12-hour-light 12-hour-dark cycle on a standard diet with food and water *ad libitum*. The four experimental groups were fed daily a standard rat chow (A04 UAR, Villemoisson sur Orge, France; ratio linoleic acid/linolenic acid = 13.1) with 30 g solid food with free access to water before the experimental transection and DHA-injection.

Study design

The experiment was performed in a fully double-blind manner: rats were coded with random numbers and the groups were mixed in cages. Experiments were undertaken in 24 rats. Animals were divided into 4 groups of 6 rats (Figure 1). Groups A and B underwent to a first surgical procedure consisting in a laminectomy (see below for procedure details) without any spinal cord transection. Group A (Control) received a placebo injection after 30 min of surgery (100 µL of physiological serum). Group B (DHA) received DHA injection (see below for fatty acid composition) 30 minutes after surgery. DHA injection consisted in intravenous, 100 µL of glycerophospholipid-liposomes obtained from DHA-enriched eggs (GPL-DHA®) (Société Application Santé des Lipides, Hauterive, France). Groups C

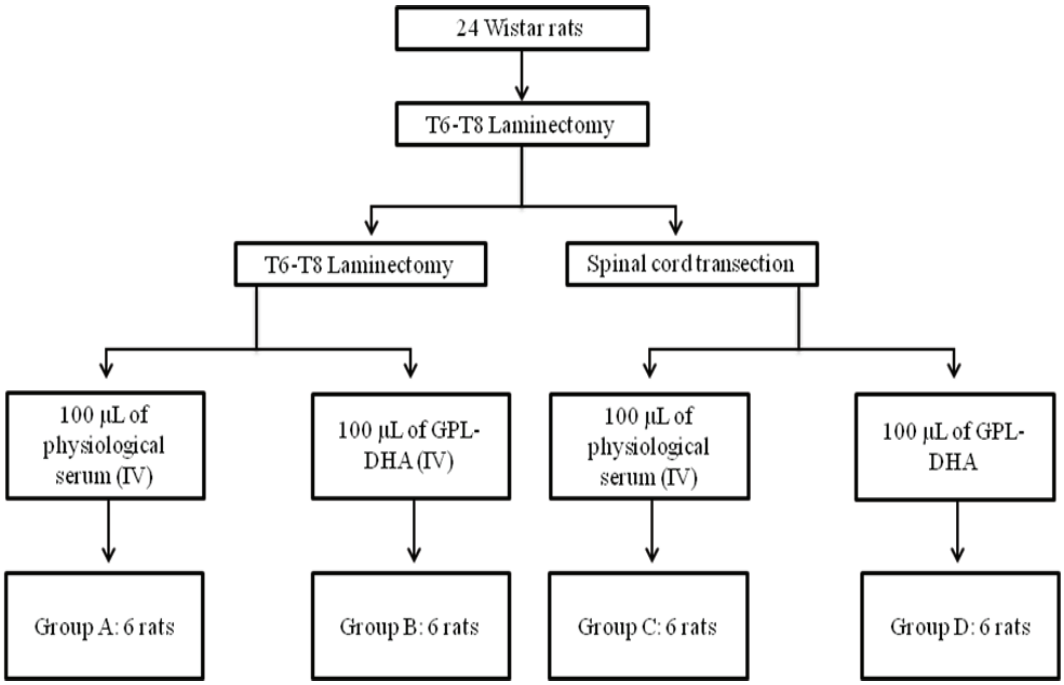

Figure 1. Study design. C: control; SCT: Spinal cord transaction; DHA: docosahexaenoic acid.

and D underwent to a surgical spinal cord transection. Group C (SCT) received a placebo injection and group D received DHA injection 30 minutes after transection (SCT+DHA).

After 3 days, distal skeletal muscle specimens, anatomically located below the transection level, were harvested to perform Na^+,K^+-ATPase activity and expression analysis.

Surgical procedures

Animals were operated on in six mixed batches and underwent an identical surgical procedure except for the spinal cord lesion. Animals were anesthetized with an intramuscular injection (3.2 mg/kg b.w.) of a solution consisting of Ketamine (3 mg/kg b.w.; Jansen Pharmaceutics, Paris, France) and chlorpromazine (1.5ml/kg b.w.; Roche Pharmaceuticals, Paris, France). Vitamin A-containing eye ointment was applied to protect the eyes from dehydration during the procedure.

Under a surgical microscope (Zeiss, Jena, Germany), the spinal cord was carefully exposed by a T6-T8 laminectomy. Groups A+B underwent the surgical procedure, including exposure of the spinal canal with laminectomy and wound closing. Spinal cord transection was performed at the previous mentioned vertebral level with an ophthalmology knife on groups C+D. We ensured there was no active bleeding in/out of the spinal canal before closing the wound. The muscles were sutured using No. 4-0 nylon, and wound clips were used to close the skin. All procedures were performed under sterile conditions. The rats were returned to cages with a warmed bedding of pine shavings, allowed to recover from surgery, and given unlimited access to food and water. After 3 days, the rats were first anesthetized to quickly collect the muscle specimens and finally sacrified by deeper anesthesia to induce cessation of respiration. Specimens consisted of biceps femoris skeletal muscle were removed, immediately frozen in liquid nitrogen, and stored at -80 °C until use.

Fatty acid composition

Eggs enriched in DHA were obtained from manipulation of the diet of the poultry by fish oil supplementation (**Table 1**). Lipid extract was obtained as already described (17), and fatty acid composition was analyzed after methylation with BF3-methanol (Sigma, St Louis, MO) by Gas Chromatography (Perkin Elmer Autosystem XL, flame ionization detector, Turbochrom software, Courtaboeuf, France) using a fused silica capillary column (Omegawax 250, 30 m x 0.25 mm i.d., 0.25 µm film thickness) (Sigma-Supelco). Hydrogen was used as carrier gas. The oven temperature program ranged from 60°C to 215°C with a temperature rise of 45°C/min. An internal standard, nonadecanoic acid, was added to the sample before methylation. Fatty acids were identified by their retention times on the column using appropriate standards (PUFA 2, Sigma-Supelco).

Muscle Plasma Membrane Isolation

Samples of frozen skeletal muscle (2-12 mg) were homogenized directly in ice-cold buffer containing 250 mM sucrose, 0.1 mM phenylmethane sulfonyl fluoride, 1 mM EDTA, and 20 mM imidazol-HCl, pH 7.4, with a polytron PT 10 (5 sec, setting 3) as previously described in cardiac tissues (23). The homogenate was subfractionated by two sequential differential centrifugations at 12,000 x g for 5 min and 540,000 x g for 5 min using a TLA100.3 rotor in the Beckman TL 100 centrifuge (Beckman Instruments; Gagny, France). The final pellet was resuspended in 250 mM sucrose and 16 mM HEPES-HCl, pH 7.4 and stored at -80C until used. These preparations consisted of a microsomal membrane fraction enriched in Na^+,K^+-ATPase.

Since Zhang and Ng (24) showed that the Na^+,K^+-ATPase α1 isoforms undergo dephosphorylation in rat skeletal muscle cryosections, we have chosen to store muscle membrane preparations in liquid nitrogen after preparation to avoid dephosphorylation.

Table 1. Fatty acid composition of DHA- enriched egg yolk powder.

Fatty acid	DHA-enriched
14:0	0.29
16:0	23.08
16:1 n-9	2.86
18:0	8.49
18:1 n-9	38.79
18:2 n-6	12.96
18:3 n-3	0.29
20:4 n-6 (AA)	0.75
20:5 n-3	0.18
22:5 n-3	0.22
22:4 n-6	0.43
22:6 n-3 (DHA)	3.70
n-6/n-3	3.39
AA/DHA	0.20

Data are presented as weight % of total fatty acids.

Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid.

K^+-stimulated paranitrophenyl phosphatase activity

Na^+,K^+-ATPase activity was measured as K^+-stimulated paranitrophenyl phosphatase (pNPPase) activity using a modified method from Maixent *et al.* (25). The activity was assessed in 96-wells plates using a reaction mixture with KCl or KCl-ouabain-NaCl during 2 h at 37°C. Each well contained (final volume 300 µl) 6 mM $MgCl_2$, 30 mM Imidazole, 250 mM sucrose, 8 mM pNPP (Sigma) with 20 mM KCl or 20 m KCl and 10^{-3} M ouabain. The complete ouabain inhibition of K^+-stimulated paranitrophenyl phosphatase (pNPPase) activity was tested after 30 min, 60 min, 90 min and 120 min. This last time of incubation was used to calculate the enzyme activity and the ouabain inhibition. The enzymatic reaction was initiated by the addition of protein (5 µg). As similar the enzyme activity's inhibition was obtained between Na^+ (20-100 mM) and ouabain, we did not choose to preequilibrate the membranes and ouabain before the addition of K^+. Optical density was measured at 405 nm using the Wallac 1420 VICTOR^{3}™ a multilabel, multitask plate reader. K^+-stimulated paranitrophenyl phosphatase (pNPPase) activity was calculated as the difference in paranitrophenyl (pNP) production with KCl or KCl-ouabain-NaCl. Enzyme activities

are expressed as optical density/hr/mg of protein. Protein content was determined by the method of Lowry et al. (26), using bovine serum albumin (Sigma) as a standard.

Antibodies

The monoclonal antibody 6F specific for the Na^+,K^+-ATPase α1 isoform was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA). The polyclonal antibody HERED specific for α2 isoform was obtained from Dr. Thomas A. Pressley (Department of Cell Physiology & Molecular Biophysic, Texas Tech University Health Sciences Center, Lubbock, Texas). The polyclonal antibody SpetB1 specific for β1 subunit was obtained from Dr. Pablo Martin-Vasallo (Laboratorio de Biologa del Desarrollo, Department of Bioquimica y Biologia Molecular, Universidad de La Laguna, Avda Astrofisico, La Laguna, Tenerife, Spain).

Western Blots analysis

The expression of α and β subunits of the Na^+,K^+-ATPase was analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 10% gel) and immunoblotting as previously described (22). In a preliminary experiment, we obtained linear calibration curves with the antibodies used in this study between 5 and 20 µg of skeletal membrane proteins (microsomal fraction) loaded on the same gel. 10 µg/lane of total protein was separated by SDS-PAGE and was transferred onto nitrocellulose membrane (Hybond C^+, Amersham Corp.) and protein expression was detected using fast western-blot (Thermo Scientific, Rockford, IL 61101 USA). The protein signals were detected with the enhanced chemiluminescence (ECL). The amount of protein bands was quantified using imageJ software (NIH).

Statistical analysis

Results are expressed as means ± SEM of six experiments. Statistical analysis was done using analysis of variance (ANOVA) followed with the Tukey's post-test (GraphPad Prism®, GraphPad Software, San Diego, CA). Values of $p<0.05$ were considered statistically significant.

Results

Na^+,K^+-ATPase activity was determined using Kpase assays (K^+-stimulated paranitrophenyl phosphatase activity) in crude membrane preparations of muscles. The results show that spinal cord transection increased significantly ($p<0.05$) Na^+,K^+-ATPase activity in muscle by 25% (Group C=SCT). Isolated GPL-DHA injection did not affect the muscle Na^+,K^+-ATPase activity (Group B=DHA). Spinal cord transection followed by an injection of GPL-DHA (Group D=SCT+DHA) affected the Na^+,K^+-ATPase activity compared to all experimental groups. In fact, Na^+,K^+-ATPase activity increased by 65%, 30% and 48% compared respectively to control (Group A), spinal cord transection (Group C) and isolated GPL-DHA injection (Group B), with a significant statistical difference ($p<0.05$) (Figure 2).

In this study, we also evaluated the levels of expression of α1, α2 and β1 -isoforms of sodium pumps

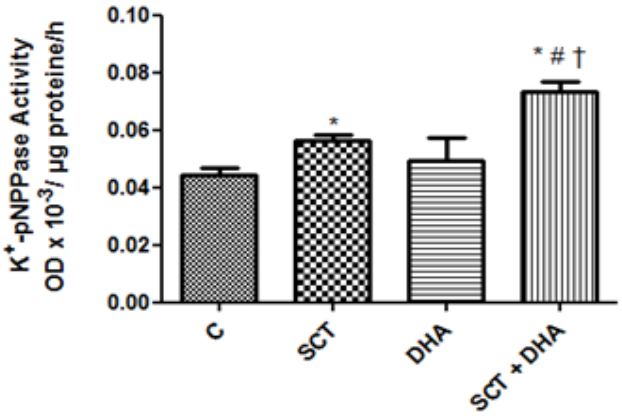

Figure 2. K^+ -stimulated paranitrophenyl phosphatase activity in plasma membrane preparation of biceps femoris skeletal muscle. Data are means±S.E.M. of six determinations. *$p<0.05$ *vs* Control, # $p<0.05$ *vs* DHA. † $p<0.05$ *vs* SCT.

in plasma membrane preparation. Western analysis, as shown in figure 3A, indicates a decreased expression of Na^+-K^+-ATPase α1 isoform in all experimental groups. It appears that Spinal cord transection, isolated GPL-DHA injection as well as early GPL-DHA administration after transection, decreased the amount of α1 isoform expression by 55%, 35% and 33% ($p<0.05$) respectively compared to controls.

In the same way, expression of Na^+-K^+-ATPase α2 was affected in our three experimental groups. Expression of α2 isoform decreased from about 50% in rat muscles between the transected group and the rats receiving GPL-DHA injection. This decrease was also observed between the transection followed by GPL-DHA injection group compared to control ($p<0.05$), but in a lower proportion (35%) (Figure 3B).

We finally assessed whether if Spinal Cord Transection, GPL-DHA or both affect Na^+-K^+-ATPase β1 subunit expression. The amount of β1 was measured in our crude muscle plasma membrane preparation, by Western blot. As depicted in figure 3C , transection followed by GPL-DHA administration caused a significant increase of β1 expression level by 25% and 40% respectively compared to control group ($p<0.05$). Injection of GPL-DHA after Spinal cord transection restored β1amount at the same level than control condition.

Discussion

In this study, the Na^+,K^+-ATPase α1, α2 and β1 protein isoform expression was altered by SCT in plasma membrane preparation from skeletal muscle. The effect of GPL-DHA administration was similar on sodium pumps, by down-regulating all Na^+,K^+-ATPase isoforms. Changes in Na^+,K^+-ATPase isoforms expression occurred within the 3 days following the lesion. These results are in accordance with the rapid effect of GPL-DHA on the cellular enrichment recently evidenced after a similar fish oil and polyunsaturated fatty acids perfusion protocol in human (14).

We also observed a specific effect of early GPL-DHA injection after SCT (Group D) at the protein isoforms Na^+,K^+-ATPase expression level and enzymatic activity of the sodium pump. An increase of the enzymatic activity of Na^+,K^+-ATPase was associated to a particu-

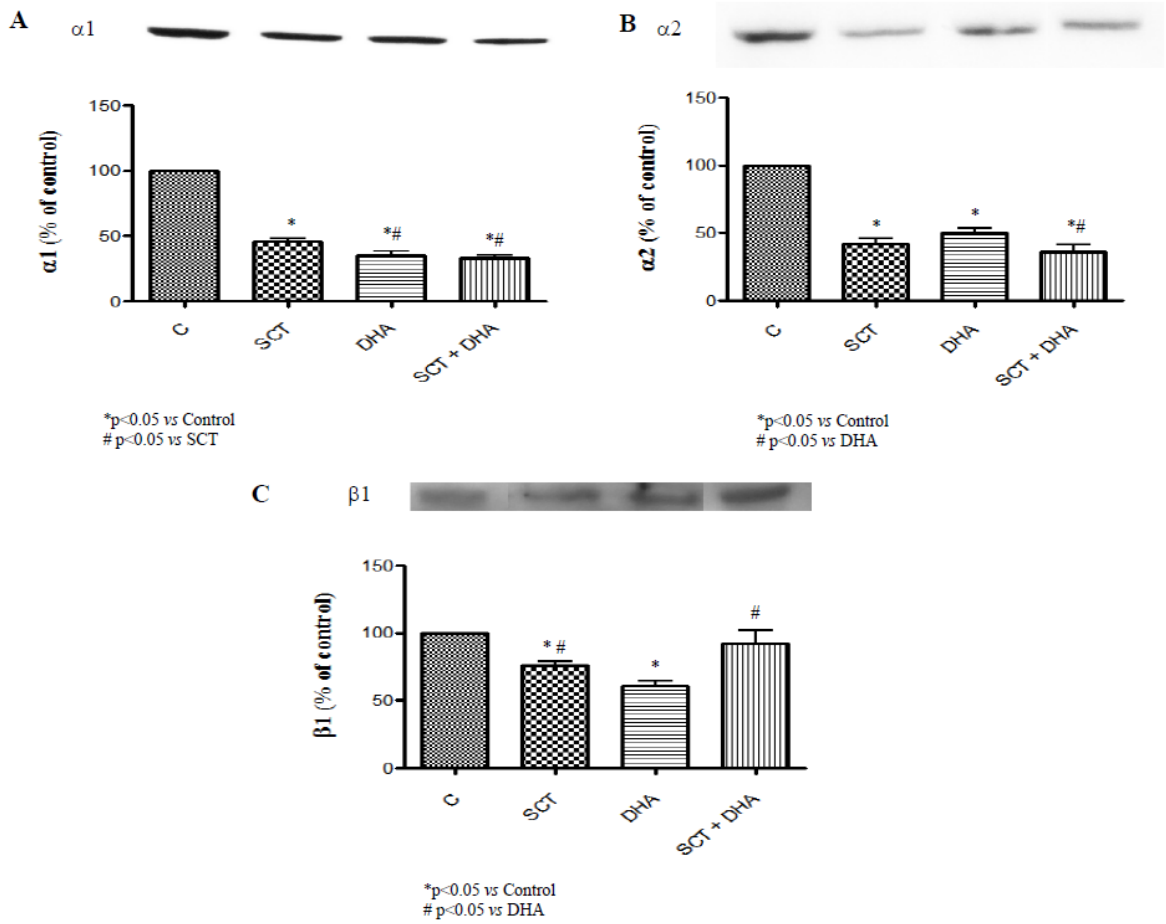

Figure 3. Na^+,K^+-ATPase α1, α2 and β1 isoforms expression in plasma membrane preparation of biceps femoris skeletal muscle. Data are means±S.E.M. of six determinations.

lar protein membrane of Na^+,K^+-ATPase β1 subunit expression. This particular expression is similar to the one found in the control group without SCT or GPL-DHA administration. To our knowledge, these results are new and original.

After SCT followed by GPL-DHA injection, the increase of activity of Na^+,K^+-ATPase appears through a Na^+,K^+-ATPase β1 subunit expression. Murphy et al. (27) have previously shown that an increase of α1, α2, and α3 mRNA can be observed without any change of β mRNA, 3 hours after electrical stimulation, in isolated rat extensor digitorum longus (EDL) muscle. The lack of electrical stimulation and consecutive membrane depolarization should lead to the opposite effect (i.e. a global decrease of α subunit expression). The decrease of β subunit-specific expression in Na^+,K^+-ATPase mRNA, described by these authors, is comparable to the effect of calcium ionophore like A-23187 in this isolated muscle (27). In our study, the muscle response to SCT or GPL-DHA may also reflect the catalytic nature of the α-isoforms, predisposing these isoforms to tight regulation imposed by changes associated with altered ion fluxes (intracellular Ca^{2+}, Na^+ and K^+ contents) (27).

In physiological conditions, muscle fibers demonstrated adaptating capabilities to ionic challenges of exercise and K^+ homeostasis, by increasing their plasma membrane Na^+,K^+-ATPase activity. Tsakiridis *et al.* (28) concluded in their report that an increased presence of α1- and α2-polypeptides at the plasma membrane and subsequent elevation of the α1 and β2 subunit mRNAs may be the mechanism by which acute exercise regulates the sodium pump of skeletal muscle. In our experimental conditions, since SCT consequences could be extrapolated to an extreme impaired exercise stretch (29), down regulation of both alpha subunit isoforms of Na^+,K^+-ATPase were also observed. However, the mRNA levels of β1 subunit expression were unaffected by this pathological condition.

Another mechanism of Na^+,K^+-ATPase regulation can occur through changes in ionic potassium (30, 31). This might also explain modulation at the α subunit of the Na^+,K^+-ATPase but in these references, such changes were also correlated to the modulation of the pump and/or enzyme membrane activity in a different way from the present study. Since the cell response may reflect the overabundance of one isoform over the others, the active enzyme complex α/β is regulated by limiting selective subunit expression in the enzyme.

In the skeletal muscle, this has been described for the β subunit and an increased expression of the α subunit or β subunit may be required for the formation of additional α heterodimers (32). A regulation through molecular turnover of the Na^+,K^+-ATPase could therefore be involved in the skeletal muscle response submitted to the experimental conditions associating SCT and DHA (33).

Recent studies demonstrated that some of the Na^+,K^+-ATPases could be involved in non pumping functions (signal transduction functions). These specific locations within caveolae are important for regulating both pumping and signal transducing functions of Na^+,K^+-ATPase (34), probably by translocation (exocytosis) and endosome internalization (endocytosis) in the skeletal muscle (35). Such a depletion of caveolae by increas-

ing the function of Na^+,K^+-ATPase (in non caveolae membranes) could be mediated through this regulation both due to SCT and DHA effects (36). Thus the signals involved in down-regulating the subunit expression of the different Na^+,K^+-ATPase isoforms appear to be complex. However, these findings are consistent with the variability of this multiple isoform expression and the different muscular fiber profiles of red or white fibers.

The possible mechanisms to explain the non parallel decrease in membrane Na^+,K^+-ATPase and Na^+,K^+-ATPase enzyme activity appear to be complex since controversies exist at the skeletal muscle for the regulation of sodium pumps (37). After SCT and DHA, the cellular traffic of Na^+,K^+-ATPase could be altered since all subunit of Na^+,K^+-ATPase are decreased in their membrane expression similar to less muscle contraction. The molecular mechanism by which the Na^+,K^+-ATPase regulation occurs ie. inhibition of the exocytosis or stimulation of endocytosis, remains to be determined. However after SCT and DHA perfusion, the modulation in the intrinsic activity of the Na^+,K^+-ATPase (estimated by Kpase activity) is essential and may involve other known substrates for Na^+,K^+-ATPase regulation such as enzyme turnover rates, post translational modification such as phosphorylation, subcellular distribution within caveolae, modulation in apparent enzyme K+ affinity, hormonal regulation and unknown skeletal muscle regulation of β_1 such as a enzyme chaperone with particular membrane locations (t-tubules).

The omega-3 and especially the polyunsaturated fatty acids such as EPA DPA, and DHA and their metabolites showed significant protective effects on health (38-40). Omega 3 and omega 6 share the same pool of elongase and desaturase (41, 42). The parent fatty acid conversion (ALA) to long chain PUFA and especially DHA occurs slowly in human and are not well understood; DHA and possible retroconversion to EPA are precursors for the formation of eicosanoids (prostaglandin, tromboxane and leukotrienes) acting as localized tissue hormones (43, 44). Their actions in regulating disease are multiple from preventing blood clotting, maintaining blood vessel patency, limiting inflammation, activating of inflammation resolution (45). All these effects could be involved in the present study to prevent the alteration induced by SCT on skeletal muscle.

The therapeutic potential of DHA after central nervous system lesion has been suggested in Michael-Titus *et al. studies* (46). An acute intravenous administration of DHA after spinal cord injury induced a neuroprotection effect by improving histological measures and reducing axonal dysfunction myelin. The loss of cytoskeletal proteins following SCI was also significantly reduced. However the consequences of neuroprotection on muscle and Na^+,K^+-ATPase has not been evidenced yet.

The α1 and α2 represent the major isoforms in adult skeletal muscles, whereas the α3 and α4 are not expressed significantly (19). It is now consensual that measurements of K^+-activated phosphatase activity are more accurate than the measures of Na^+,K^+-ATPase enzyme activity in purified plasma membranes from skeletal muscles. This is due to the concomitant presence of Sarcolemma reticulum (SR) Ca^{2+}-ATPase (SERCA) in skeletal muscle, which contaminates the measure of the Na^+,K^+-ATPase activity. A close relationship between the activity and the total pump content and isoform content of Na^+,K^+-ATPase has been clearly demonstrated (37). We have investigated the very short term effects (less than one hour) of EPA and DHA on the Na^+,K^+-ATPase activity previously, by incubating human endothelium microsomal fractions with various free DHA (47). Under this *in vitro* condition, DHA reduced Na^+,K^+-ATPase enzyme activity. This effect corresponds to a direct effect of DHA on the membrane and/or the Na^+,K^+-ATPase. The same experiment with EPA-DHA from fish oil showed some neuroprotection effects on induced diabetes, decreasing the Na^+,K^+-ATPase enzyme activity (48). A comparable cardioprotection was observed with fish oil (21) against the digitalis induced contractures and altered cell energetic metabolism. The relationships between the prevention of the Na^+,K^+-ATPase enzyme over-activation and the down-regulation of alpha subunit isoform expression were correlated. Moreover, all these effects were found again in an other chronic organ disease after two months of a continuous fish oil or DHA diet (18). The results obtained here show that an intravenous DHA bolus clearly dissociates the membrane enzyme activity from its subunit expression. The DHA action mechanism could be the resultant of the different protection mechanisms previously described. Diets with PUFA were shown to affect membrane PUFA composition of rat skeletal muscle (49). Subsequently, the increase of membrane DHA levels after intravenous DHA infusion could be the main underlying mechanism by which DHA is acting on the muscle. Recently, as EPA and DHA diets for months induced it, we found that an intravenous bolus injection of a fish oil triacylglycerol emulsion in human allowed rapid enrichment in leukocytes and platelet phospholipids within 60 min and ≥ 24 h after the injection (14). For this study, we used a specific molecular form, phospholipids of DHA that could affect the cell differently from the previous chronic studies. We tested the effect of a intravenous bolus injection with an emulsion of mainly DHA linked to phospholipids (16). This vector seems to be more resistant to oxidation with more cell membrane DHA accretion when compared to triacylglycerol or ethyl esters forms (17). Thus we could speculate that a change occurring in the plasma and skeletal membranes fatty acid composition could be the result of a single isolated DHA bolus infusion as previously described (50). Future human studies are needed to confirm this hypothesis.

In summary, this study demonstrated that spinal cord transection induces an alteration of Na^+,K^+-ATPase subunit isoform expression in skeletal muscle, similarly to isolated GPL-DHA bolus infusion. The administration of GPL-DHA after a spinal cord transection increases the membrane Na^+,K^+-ATPase activity in skeletal muscle and seems to prevent the β1 subunit of the Na^+,K^+-ATPase alteration. The overall increase in expression of β1 subunit could be described as the trigger to the sodium pump reactivation in the skeletal muscle after a trans-synaptic denervation.

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M.M, C.F., and M.F., M.F., C.F., and A.D. conducted the experiments. Application Santé des Lipides, ASL, (Bioparc de Vichy, Hauterive, France) produced the egg powder-enriched in DHA. Dr Thierry Coste and Gérard Pierroni (ASL) analyzed the fatty acids. Statistical analysis was conducted by M.F and C.F. Data were interpreted by J-M.M., P.R., M.F., A. D., M.F., C.F. and J-M.M were responsible for the writing of the manuscript. The manuscript was reviewed and approved by all authors.

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