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Skeletal Muscle Atrophy in Men with Chronic Alcoholic Myopathy (impairment to signal pathways)

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The main effect of chronic alcoholic myopathy is atrophy of fast muscle fibers (18 years of alcohol abuse), followed by slow fibers (31 years of alcohol abuse); plasma IGF-1 levels decrease and muscle IRS-1 and p-p70S6k decrease, which is evidence for suppression of the functioning of the mTORC1 signal pathway and reductions in protein synthesis. Patients show increases in the expression of mRNA encoding HSP90/70, which can lead to increases in the protection of proteins from degradation. All patients showed increased expression of E3 ligase, which is evidence for an increase in the operation of the ubiquitin-proteasomal signal pathway for protein degradation.

Keywords: chronic alcoholic myopathy, E3 ligase, intracellular signal pathways.

Patients with chronic alcoholic myopathy (CAM) show hypotrophy of the muscles of the lower limbs, impaired gait, decreased work capacity, and muscle weakness. The pathogenesis of this disease has received little study despite its prevalence. Muscle atrophy (induced by hypokinesia or other diseases) can develop as a result of impairment to a number of signal pathways controlling protein synthesis or degradation. These targets are different in different diseases. In the case of myopathy induced by prolonged alcohol intoxication of patients, no studies have determined which are the main signal pathways impaired. Investigation of this question should allow therapeutic measures to be developed as the disease is widespread and has high social significance. Previous experiments modeling alcohol consumption in animals showed that in alcoholic myopathy, the signal pathways controlling protein synthesis are more impaired than those supporting protein degradation [16]. We report here the first studies of changes in anabolic and catabolic signal pathways in the vastus lateralis muscle in male patients with CAM and long (18 and 31 years) periods of alcohol abuse with the aim of identifying the signal pathways most sensitive to the actions of ethanol to provide for studies of the mechanisms by which this socially significant disease develops and to support the development of methods of prophylaxis.

Methods. Patients with chronic alcohol intoxication under hospital treatment at the Kozhevnikov Nervous Diseases Clinic were studied. Studies involving patients with alcohol intoxication were approved by the Ethics Committee (Protocol 0908 of the Interinstitutional Ethics Committee, November 13, 2008). Patients were divided into two groups based on the duration of alcohol abuse: 18.1 ± 1.2 years (group 1, $n = 7$) and 31.3 ± 1.0 years (group 2, $n = 7$); mean age was 47.7 ± 2.0 years. The reference group consisted of seven healthy male volunteers (39.7 ± 5.0 years, control group). All signed informed consent to take part in the study. The daily alcohol dose was the same in the two groups of patients and the mean dose was 16.1 ± 1.4 units of alcohol (which according to the WHO criteria imposes a high risk of developing the complications of alcohol intoxication). Definition: 1 Unit is 10 ml of pure (96%) alcohol. All subjects of the control group denied abuse of spirits and at the time of the study did not have acute and/or chronic diseas-

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es or signs of peripheral nerve or skeletal muscle damage. Patients and control group volunteers underwent clinical investigation to exclude concomitant diseases. Biopsies of the vastus lateralis muscle were taken from all patients and volunteers; tissue was frozen in liquid nitrogen and then stored at –85°C.

Immunohistochemical studies of the vastus lateralis muscle used primary antibodies to myosin heavy chains (MHC) types I and II from Sigma (USA) diluted 1:500 and 1:300, respectively, with Alexa Fluor 488 (1:500) secondary antibodies from Thermo Fisher Scientific (USA). Preparations were photographed at a magnification of ×400 using a Leica Q500MC microscope (Germany) and a Leica DC300F camera (Germany). The numbers of fibers expressing different isoforms of MHC were counted and the mean cross-sectional areas of muscle fibers (CSA of MF) were measured using LeicaQwin. Relative contents of the fast and slow MHC isoforms were determined and mean CSA of MF calculated using at least 100 fibers of each type.

Anabolic signaling marker contents were determined by electrophoresis and western blotting. A cryostat was used to cut sections of vastus lateralis muscle tissue of thickness 20 μm which were then placed in cooled RIPA Lysis Buffer System (supplemented with 0.5 M EDTA 24 μl/ml, Na_3VO_4 20 μl/ml, DTT 4 μl/ml, PMSF 20 μl/ml, aprotinin 5 μl/ml, leupeptin 5 μl/ml, pepstatin A 5 μl/ml, Phosphatase Inhibitor Cocktail B 40 μl/ml) at a rate of 130 μl of buffer per sample. Tissue was then homogenized and centrifuged (15 min, +4°C, 12000 rpm). Protein concentrations in lysates were determined by the Bradford method. Laemmli electrophoresis was run in 10% separating polyacrylamide gels in a Bio-Rad minisystem (USA) for 1 h at a current of 17 mA per gel. Proteins were transferred to nitrocellulose membranes (Bio-Rad, USA) using a mini Trans-Blot apparatus (Bio-Rad, USA) for 2 h at a temperature of +4°C and a constant voltage of 100 V. For IRS-1, 7.5% separating gels were used and transfer was for 90 min at a current of 0.35 A. Uniform protein loading of all lanes was verified by staining the nitrocellulose membrane with Ponceau S. After washing to remove stain, membranes were blocked in 5% dried milk in PBST (PBST + 0.1% Tween 20) at room temperature for 1 h. Membranes were then incubated (15 h, +4°C) with primary antibodies against IRS-1 (1:500), p-p70S6k (Thr389) (1:2000), and pAMPK (Thr172) (1:500) from Santa Cruz Biotechnology (USA). After washing, membranes were incubated for 1 h with secondary antibodies to rabbit immunoglobulins (Santa Cruz, USA) diluted 1:1000. GAPDH was detected using secondary antibodies to mouse immunoglobulins diluted 1:20000 (Bio-Rad, USA). Bands were detected on membranes using a Star TM Substrate Kit (Bio-Rad, USA). Protein bands were analyzed using a C-DiGit blot scanner (LI-COR Biosciences, USA) and the program Image Studio Digits (LI-COR Biosciences, USA).

Real-time PCR studies were performed to determine the levels of mRNA encoding E3 ligases MuRF-1 and Atrogin-1 and heat shock proteins 70 and 90. Total RNA was extracted for reverse transcription from 10 mg of frozen soleus muscle using an RNeasy Micro Kit (Qiagen, Germany). All RNA samples were treated with proteinase K and DNase I. RNA concentrations were determined at a wavelength of 260 nm. Reverse transcriptase was performed using a solution containing 1 μg of RNA, oligo(dT)15, random hexanucleotides d(N)6, and MMLV reverse transcriptase. Reverse transcription was run at 37°C for 60 min using a standard protocol. The following primers were used: 5'-ggctgctgtggaagaaactc-3' and 5'-ccttccaggaaaggatgtga-3' for MAFbx; 5'-tgagccagaagtttgacacg-3' and 5'-gatgagttgcttggcagtca-3' for MuRF-1; 5'-cgcatgaaggagacacagaa-3' and 5'-tcccatcaaattccttgagc-3' for HSP90β; 5'-ccgagaaggacgagtttgag-3' and 5'-aatcttggaaaggcccctaa-3' for HSP70. All samples were analyzed at least three times and all reactions were monitored using an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, USA); GAPDH was used as the reference gene.

Gene expression data were analyzed statistically in REST 2009v software. Western blotting data were analyzed in Origin Pro v.8.0 SR5. Groups were compared using the Mann–Whitney test. PCR results are given as medians and interquartile widths (0.25–0.75) and western blotting results as means and errors of the mean.

Results and Discussion. *Morphological changes in muscles in alcohol abuse*. Groups 1 and 2 demonstrated muscle fiber atrophy as compared with the control group. While CSA decreased only in fast MF in group 1 ($p < 0.05$), group 2 showed decreases in CSA in both types of MF ($p < 0.05$) (see Table 1). The proportion of fast MF increased in all patients and the proportion of slow MF decreased (see Table 1, $p < 0.05$) as compared with the control group. It is interesting to note that the literature contains descriptions of cases of decreases in CSA of MF of only the locomotor muscles expressing the "fast-type" myosin II isoforms in chronic alcohol administration [4, 7]. Atrophy of the "slow" soleus muscle in alcoholic rats has not previously been noted [15] (no such studies have been performed in humans). Furthermore, some authors have shown that fibers of the first type are not affected by ethanol [3]. Our findings indicate that this is not so, and that CSA of type I MF also undergo atrophy on prolonged alcohol exposure. We have previously reported an increase in the percentage content of fast MF in patients with CAM [1]. No such data are present in the world literature because of the small number of invasive studies of muscles in humans.

Operation of signal pathways controlling the processes of protein synthesis in skeletal muscles in alcoholism. Decreases in muscle mass occurred as a result of impairment of the balance between the rates of protein synthesis and degradation. Decreases in the content of the anabolic agent IGF-1 (insulin-like growth factor) in plasma were seen in both groups of patients relative to the control group (by 56% and 51%, respectively, see Table 1). IGF-1

TABLE 1. Cross-Sectional Areas, Percentage Ratios of Types of Muscle Fibers, Plasma IGF-1 Contents, and Levels of Expression of HSP70, HSP90, MuRF 1, and MAFbx in the Vastus Lateralis Muscle in the Control Group and Patients with CAM

Parameter	Control	Group 1	Group 2
CSA of MF I, μm^2	4111 ± 353	3886 ± 389	2970 ± 149*
CSA of MF II, μm^2	4073 ± 288	3088 ± 249*	2490 ± 244*
MF I, %	38.8 ± 5.2	31.5 ± 2.0	29.0 ± 3.0*
MF II, %	41.3 ± 2.8	56.6 ± 2.4	63.0 ± 2.0*
Hybrid MF, %	19.8 ± 4.0	11.8 ± 2.6	8.0 ± 3.0
IGF-1, ng/ml	216 ± 63.6*	94.8 ± 7	106.3 ± 9.2
mRNA MuRF-1	1.00 (0.2–0.5)	7.00* (1.9–13.5) $p = 0.05$	3.73* (1.0–7.5) $p = 0.048$
mRNA MAFbx	1.00 (0.12–9.5)	5.91* (1.1–16.0) $p = 0.026$	3.83 (0.7–11.0) $p = 0.113$
mRNA HSP70	1.00 (0.25–8)	12.29* (1.6–110) $p = 0.031$	14.00* (1.6–110) $p = 0.013$
mRNA HSP90	1.00 (0.19–7.0)	9.14* (2.35–33.5) $p = 0.009$	7.4* (1.9–26.5) $p = 0.015$

*Significant differences from control group, $p < 0.05$.

is involved in the endocrine, autocrine, and paracrine regulation of growth and development processes; decreases in its plasma concentration in alcoholics have been reported previously [11]. Most studies addressing signal pathways which might be impaired in alcohol intoxication have been performed using models of alcoholization in animals. Many fewer studies of human subjects have been reported. Do decreases in plasma IGF-1 levels affect the rates of anabolic processes in skeletal muscles? IGF-1 receptors play the key role in transmission of signals to IRS-1 (the substrate of the insulin receptor-1) and to anabolic PI3K/Akt/mTORC-1 (rapamycin-dependent) and ERK1/2 (mitogen-activated) signal pathways. We found significant decreases in IRS-1 in both groups of patients (by 24% and 40%, respectively, Fig. 1) relative to the control groups. Decreases in IRS-1 levels have been seen previously in muscles in alcoholized rats [5]. We also observed decreases in p-p70S6k levels (ribosomal S6 protein kinase) in both groups of patients with CAM, by 25% and 30%, respectively, compared with the control group ($p < 0.05$, Fig. 2). This kinase is a key marker for the rapamycin-dependent signal pathway, which is involved in initiating translation. Protein synthesis in skeletal muscle is regulated mainly by the mTORC-1 signal cascade, which stimulates phosphorylation of its main substrates: S6 kinase (S6K) and eukaryotic translation initiation factor 4E-binding protein-1 (4E-BP1) [6]. Korzick et al. reported decreased phosphorylation of p70S6K in old alcoholized rats [10]. We have previously noted a decrease in p-4E-BP1 in patients after seven years of alcohol abuse [2]. Phosphorylation of this factor is known to inhibit initiation of protein translation. Lang et al. (2003) reported decreased protein synthesis in muscles due to suppression of mTOR-dependent translation initiation in experiments on alcoholized rats [8, 12, 13].

We did not find any significant changes in pAMPK (phospho-5-AMP-activated protein kinase) in any group of patients. AMPK controls the energy balance of the cell [9]. Phosphorylation of AMPK usually increases when operation of insulin-stimulated IRS-1 is suppressed. In our case, IRS-1 was decreased in male patients. This pathway is generally activated in metabolic syndrome to increase the operation of GLUT4 (the glucose utilization shunt). We have previously seen increases in pAMPK in patients with CAM with seven years of alcohol abuse [2], but it is likely that pAMPK levels return to the control group level as the duration of alcoholization increases.

Operation of signal pathways controlling protein degradation processes in skeletal muscles in alcoholism. The view that signaling systems initiating protein degradation in alcohol abuse are afflicted to a lesser extent than those initiating synthesis is widely held [8]. Protein degradation is regulated by the calpain, ubiquitin-proteasomal, and autophagic-lysosomal signal systems. We found increases in the expression of E3 ligases MuRF-1 and MAFbx/Atrogin-1 in the vastus lateralis muscle in male patients at all stages of the development of chronic alcohol intoxication (Table 1). Some investigators have previously observed increases in the content of the mRNA species encoding these E3 ligases in the muscles of alcoholized rats [10]. MuRF-1 and MAFbx are usually used as markers for ubiquitin-proteasomal activity, as they mediate the conjugation of ubiquitin with target proteins. We have previously demonstrated increases in protein ubiquitinylation in muscles in both men and women after 5–7 years of alcohol abuse [2]. Thus, atrophy of the

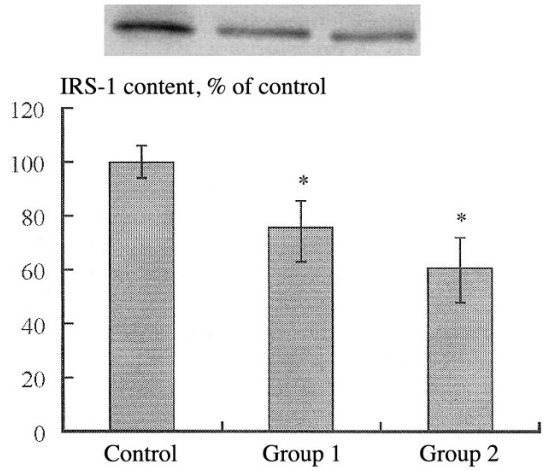

Fig. 1. Total IRS-1 level in the vastus lateralis muscle in patients (relative to control group). Group 1 – patients consuming alcohol for 18 years; Group 2 – patients consuming alcohol for 31 years. Above histogram – typical western blot of IRS-1 protein typical of each group. *Significant differences from control group, $p < 0.05$.

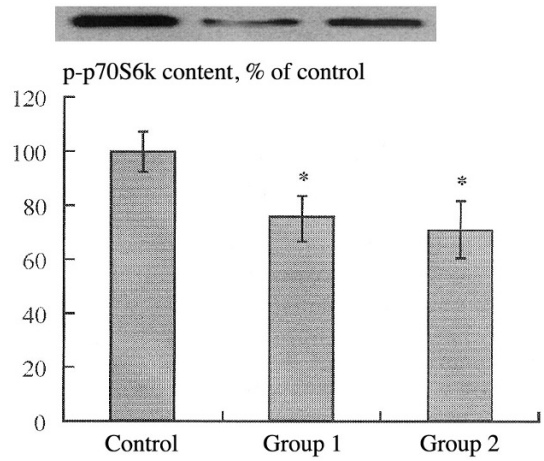

Fig. 2. Phosphorylated p-p70S6k levels in the vastus lateralis muscle in patients (relative to control group). Group 1 – patients consuming alcohol for 18 years; Group 2 – patients consuming alcohol for 31 years. Above histogram – typical western blot of p-p70S6k protein typical of each group. *Significant differences from control group, $p < 0.05$.

vastus lateralis muscle is accompanied by increases in protein degradation processes.

Expression of heat shock proteins in skeletal muscles in alcoholism. We found an increase in the expression of mRNA encoding heat shock proteins Hsp70 and Hsp90 in male patients (Table 1). This increase in expression may be a protective reaction to the protein degradation and atrophic processes seen in these patients. Previous studies have shown that heat shock proteins can protect proteins from proteolysis [14].

Conclusions

1. Prolonged alcohol abuse (18 years) leads to decreases in the CSA of type II MF, while longer periods of abuse (31 years) result in atrophy of types I and II MF.

2. All patients with CAM experienced decreases in plasma IGF-1, p-IRS-1 and p-p70S6k contents, which is evidence for suppression of the operation of the mTORC1 signal pathway and reductions in muscle synthesis in muscles.

3. Patients of both groups showed increases in the expression of HSP90/70 mRNA which may lead to increases in the protection of proteins against degradation.

4. All patients with CAM showed increases in the expression of E3 ligases, which is evidence for increased operation of the ubiquitin-proteasomal protein degradation signal pathway.

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