INFLUENCE OF DIAGNOSTIC CATEGORIES, AGE, AND GENDER ON ANTIOXIDATIVE DEFENSE AND LIPID PEROXIDATION IN SKELETAL MUSCLE OF PATIENTS WITH NEUROMUSCULAR DISEASES

Hans-Joerg Stuenerburg, Birgit Stangneth, Alfried Kohlschütt, and Barbara Finckh
Departments of *Neurology and t Pediatrics, University of Hamburg Hamburg, Germany

ABSTRACT
The influence of diagnostic categories, age, and gender on parameters of oxidative stress measured in 102 patients with neuromuscular diseases and 11 control subjects was assessed using a stepwise multiple linear regression model. Antioxidative enzyme activities, lipophilic antioxidants, and lipid peroxidation were analyzed in muscle biopsies.

Mitochondrial myopathies and amyotrophic lateral sclerosis (ALS) are thought to be particularly susceptible to increased oxidative stress. In our study, mitochondrial myopathies emerged as a positive predictor of malondialdehyde (p < 0.05) and ALS as a negative predictor of alpha-tocopherol (p < 0.05). Although the primary atrophic process in ALS is not in muscle but in motoneurons, this finding could have therapeutic implications, as such patients might benefit from antioxidant supplementation.

In our study age emerged as a negative predictor of the coenzyme Q10 concentration (p < 0.003), whereas the percentage of reduced coenzyme Q10 remained unchanged. Age emerged as a positive predictor of the activities of catalase (p < 0.01) and superoxide dismutase (p < 0.002), probably reflecting an enzymatic upregulation that compensates for the loss of coenzyme Q10. The increased activities of catalase and superoxide dismutase in females compared to males indicate a higher antioxidative potential in female muscle. Whether this increase contributes to a higher life expectancy of women remains to be investigated.

INTRODUCTION
Oxidative stress is involved in the physiological process of aging and probably in the pathophysiology of several human neuromuscular diseases. However, data on antioxidative potential and free radical damage in human muscle are sparse due to lack of biopsy material (1-3). In particular, peroxidation of polyunsaturated fatty acids in cell membranes and oxidative damage to DNA probably contribute to free radical damage (2,4,5). In human skeletal and cardiac muscle, mitochondrial DNA deletions and the content of 8-hydroxy-2-deoxyguanosine as a marker of oxidatively degraded mitochondrial DNA increase with age (6-8). The antioxidative defense of human muscle consists of lipophilic and hydrophilic antioxidants such as tocopherol, coenzyme Q10, and vitamin C, and of protective enzymes such as glutathione peroxidase, catalase, and superoxide dismutase. The enzyme activities of glutathione peroxidase, catalase, and superoxide dismutase are elevated in skeletal muscle of aged rats (9). The antioxidative defense system is probably in dynamic equilibrium, where the decrease in one factor can be compensated by the upregulation of another (10).

The cause or the progression of certain neuromuscular diseases may be influenced by free radicals, one particular example being motoneuronal damage in the familial form of amyotrophic lateral sclerosis (ALS), in which part of the patients carry mutations in the Cu/Zn-SOD-gene (11). In ALS, however, processes initiated by reactive oxygen species probably result in primary damage to motoneurons, while the damage to skeletal muscle is a secondary event (12). Several studies describing increased concentrations of protein carbonyls and 3-nitrotyrosine in spinal cord tissue provide evidence for the implication of oxidative damage in disease pathogenesis of ALS (for review see (13)).

The objective of the present study was to analyze antioxidative status and lipid peroxidation in muscle biopsy specimens from controls and from patients with different neuromuscular diseases, with particular emphasis on changes that manifest during aging. The biochemical parameters were measured using micromethods and calculated by multifactorial statistical analysis including age, gender, and diagnostic category as independent variables.

PATIENTS AND METHODS

Patient groups
Biopsy specimens from 113 patients with neuromuscular diseases were analyzed. The diagnostic categories, age, and gender of these patients are shown in Table 1. A group of patients diagnosed to have no neuromuscular diseases (n = 11) was used as a control group.

Tissue preparation
Muscle biopsies (mainly from musculus tibialis anterior) were taken for diagnostic reasons from patients with suspected neuromuscular diseases after informed consent. Small pieces of tissue (50 - 100 mg) obtained during biopsy and subsequent processing were immediately frozen in liquid nitrogen, as is also done with tissue intended for histological processing, and kept at -80°C until analysis. No additional tissue was taken for the study presented here. The muscle biopsy specimens were cut into small pieces, pulverized under liquid nitrogen, and the...
tissue powder was used for determination of antioxidative status and lipid peroxidation as Fig. 1 demonstrates. Homogenates (1:5, weight/volume) in phosphate-buffered saline were prepared and centrifuged (20,000 g, 15 min, 4°C), and the supernatant (cytosol) was used for measurement of glutathione peroxidase, catalase, and superoxide dismutase (14). Other homogenates (1:40, w/v) were prepared for measurement of malondialdehyde, alpha-tocopherol, and reduced and oxidized coenzyme Q\(_{10}\). Homogenization buffer (10 mM potassium phosphate (pH 7.4) containing 30 mM KCl, 10 mM diethylene-triaminepentaacetic acid, and 100 KIU/mL aprotinin) according to Hübner et al. was used, and butylhydroxytoluol (57 mM in ethanol; 2 μL/mg muscle tissue) was added to the preparation (15). All procedures for preparation of the muscle biopsy homogenates were performed in a cold storage room (4°C).

**Lipid peroxidation**

Malondialdehyde was measured in 10 μL homogenate (1:40, w/v) in triplicate using HPLC with fluorescence detection (16).

**Antioxidative enzymes**

The activity of selenium and non-selenium-dependent glutathione peroxidase was analyzed in diluted cytosol (1:10; w/v) with cumolhydroperoxide as starting reagent in the assay (17). The activity of catalase in diluted cytosol (1:70; w/v) was analyzed after activation (1% ethanol, 0.01% Triton-X-100) by measuring decomposition of H\(_2\)O\(_2\) (14). The activity of superoxide dismutase in 50 μL cytosol (1:40; w/v) was analyzed by measuring the superoxide dismutase-mediated increase in the rate of autooxidation of BXT-01050 (5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo(c)-fluorene) to a chromophore (18).

**Lipophilic antioxidants**

Alpha-tocopherol and coenzyme Q\(_{10}\) (reduced/oxidized) were measured in 40 μL homogenate (1:40, w/v) after addition of 20 μL lauryl sulfate (0.1 M) using HPLC with electrochemical detection and internal standardization with gamma-tocotrienol and reduced and oxidized coenzyme Q\(_{9}\) (19).

**Protein and total lipids**

The protein contents in cytosol and homogenates and the total lipid content in homogenates were measured using routine methods (20, 21).

**Statistical analysis**

The data were analyzed with the Statview II statistical package (Statview II, Abacus Concepts, Inc., Berkeley, CA). A stepwise multiple linear regression model was used to assess the influence of the independent variables (diagnostic category, age, and gender) on the dependent variables. The individual diagnostic categories were introduced as indicator variables and the parameters measured were entered as dependent variables of continuous type into the multiple stepwise regression model (step in, step out, F to enter = 4.0; F to remove = 3.996). Statistical significance was set at the 0.05 level.

### RESULTS

**Lipid peroxidation**

For the diagnostic categories of mitochondrial myopathy and polyneuropathy, elevated malondialdehyde concentrations were detected compared to controls as Table 2 demonstrates. Age and gender were no significant predictors of the malondialdehyde concentration.

**Antioxidative enzymes**

No differences were found in the enzyme activities of glutathione peroxidase, catalase, and superoxide dismutase for the diagnostic categories versus controls (Table 2). The age of the patients emerged as a significant positive predictor of the enzyme activities of catalase (Fig. 2A) and superoxide dismutase (Fig. 2B), but not for the enzyme activity of glutathione peroxidase. The theoretical changes between 20 and 80 years of age (calculated by simple linear regression analysis) were +58.8 % for the catalase activity and +98.9 % for superoxide dismutase activity. The gender of the patients turned out to be a significant predictor of catalase activity (male: 44.0 ± 2.7, female 51.8 ± 2.4 μmol/mg protein x min; mean ± standard error of the mean (S.E.M.); p<0.05) and superoxide dismutase activity (male: 2.7 ± 0.2, female 3.3 ± 0.2 U/mg protein; mean ± S.E.M.; p<0.05).

**Lipophilic antioxidants**

For the diagnostic category of ALS, reduced alpha-tocopherol concentrations were found compared to controls (Table 2). There was no significant predictor of alpha-tocopherol in relation to the total lipid content of the muscle. Age and gender of the patients were no significant predictors of the concentration of alpha-tocopherol. Gender and diagnostic categories were no predictors of the total coenzyme Q\(_{10}\) concentration per total lipids. Moreover, there were no significant predictors for either the total coenzyme Q\(_{10}\) concentration relative to protein content, or for the reduced coenzyme Q\(_{10}\) / total coenzyme Q\(_{10}\) ratio. Age was a significant negative predictor of the total coenzyme Q\(_{10}\) concentration relative to total lipid content (Fig. 3). The theoretical change between 20 and 80 years of age (calculated by simple linear regression analysis) was -42.7 %.

**DISCUSSION**

We measured the antioxidative status and lipid peroxidation in small muscle biopsy specimens from patients with neuromuscular diseases. Increased levels of malondialdehyde in muscle tissue of patients with mitochondrial myopathies could be explained by the increased generation of free radicals without a compensating increase in concentrations of the lipophilic antioxidants alpha-tocopherol and coenzyme Q\(_{10}\), or in activities of the antioxidative enzymes (22). Moderately increased levels of malondialdehyde were shown in muscle tissue of patients with polyneuropathies. A common feature shared by malondialdehyde and diabetic neuropathies, is an underlying metabolic disorder accompanied by poly-