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

Hans-Joerg Stuerenburg,* Birgit Stagneth,† Alfried Kohlschütter,† and Barbara Finckh †

Departments of *Neurology and † 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 Q_{10} concentration ($p < 0.003$), whereas the percentage of reduced coenzyme Q_{10} 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 Q_{10} . 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 Q_{10} , 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 micro methods 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

To whom all correspondence should be addressed:

Dr. Barbara Finckh
Universitätsklinikum Hamburg-Eppendorf
Klinik für Kinder- und Jugendmedizin
Martinistr. 52
20246 Hamburg, Germany
Tel.: int+40-42803-2737
Fax.: int+40-42803-5984
e-mail: b.finckh@uke.uni-hamburg.de

tissue powder was used for determination of antioxidative status and lipid peroxidation as Fig. 1 demonstrates. Homogenates (1:5, weight/volume (w/v)) 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 Q₁₀. Homogenization buffer (10 mM potassium phosphate (pH 7.4) containing 30 mM KCl, 10 mM diethylenetriaminepentaacetic 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 H₂O₂ (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 Q₁₀ (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 Q₉ (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 $\pm$ 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 $\pm$ 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 Q₁₀ concentration per total lipids. Moreover, there were no significant predictors for either the total coenzyme Q₁₀ concentration relative to protein content, or for the reduced coenzyme Q₁₀ / total coenzyme Q₁₀ ratio. Age was a significant negative predictor of the total coenzyme Q₁₀ 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 Q₁₀, 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 the heterogeneous group of polyneuropathies, which comprises, for example, alcoholic neuropathies and diabetic neuropathies, is an underlying metabolic disorder accompanied by poly-

neuropathy as a secondary symptom. These underlying metabolic disorders may be responsible for the increased malondialdehyde levels.

None of the diagnostic categories emerged as predictors of the activities of the antioxidative enzymes glutathione peroxidase, catalase, and superoxide dismutase, or the lipophilic antioxidant coenzyme Q_{10} , whereas the diagnostic category of amyotrophic lateral sclerosis (ALS) turned out to be a predictor of alpha-tocopherol. The concentration of alpha-tocopherol was reduced in muscle biopsy specimens from these patients. Although the primary atrophic process does not take place in skeletal muscle but in motoneurons, reduced alpha-tocopherol levels might influence disease progression. A reduction in alpha-tocopherol could reflect an increased consumption in association with increased oxidative stress in ALS patients, or an altered intake due to a change in the nutritional situation. In any case, the lowered antioxidative capacity in skeletal muscle mirrored by reduced alpha-tocopherol is of possible clinical and therapeutic significance, because substitution of alpha-tocopherol is possible.

In our study, no age-associated increase in lipid peroxidation was found in muscle tissue. This result is in agreement with previous animal studies in which no age-associated increase in malondialdehyde was observed (23), but it is in disagreement with the studies of Mecocci *et al.* and Pansarasa *et al.* (1,2). Their results showed higher levels of lipid peroxidation products in two older age groups (age: 66-75 years, 76-85 years) in comparison to a young age group (age: 17-25 years). This discrepancy may be due to a different approach in handling data.

The age of the patients was a positive predictor of the activities of the antioxidative enzymes catalase and superoxide dismutase. The activities increased with age. For glutathione peroxidase, though, there was no correlation between enzyme activity and age. These results are in agreement with previously published data obtained in rats, in which increases in activities of antioxidative enzymes, in particular of catalase and superoxide dismutase, were found in various types of skeletal muscle during aging (24). This suggests that the age-associated increase in catalase and superoxide dismutase activities in muscle tissue of our patients is an adaptation to increasing oxidative stress in muscle. This correlation between antioxidative enzyme activity and aging probably accounts for the fact that we did not detect an elevation in lipid peroxidation with increasing age. In the study of Fano *et al.*, there was no difference between the glutathione peroxidase activity in skeletal muscle of a young and an old age group. In contrast to our study, they found no difference in SOD activity and a decrease in catalase activity (25). In addition, a comparison between the old and young age group showed an increase in lipid peroxidation in skeletal muscle. Whether this discrepancy is due to the different approach in data handling or to the differences in study population (for example nutrition) remains unclear.

The increased enzyme activities of catalase and superoxide dismutase in the female group compared to the male group indicate that there is a higher

antioxidative potential in the skeletal muscle of females. In agreement with this, Fano *et al.* showed lower levels of 8-hydroxy-2-deoxyguanosine and malondialdehyde in the skeletal muscle of elderly females compared to elderly males (25). The question to what extent a higher antioxidative potential in female muscle is linked to a higher life expectancy of women remains to be answered.

With increasing age, a marked decrease was shown for the concentration of total coenzyme Q_{10} in skeletal muscle. In combination with our finding that the ratio of reduced coenzyme Q_{10} to total coenzyme Q_{10} is unchanged with age, these data demonstrate a decrease in antioxidative potential caused by decreased levels of reduced coenzyme Q_{10} (26). The plasma concentrations of coenzyme Q_{10} in humans decrease significantly with increasing age. Likewise, a decrease in coenzyme Q_{10} was shown in rat heart and other tissues (26,27). The aged myocardium is clearly more sensitive to aerobic and ischemic stress in humans and in animals, and coenzyme Q_{10} treatment before ischemia markedly improves recovery (28,29). Also, a diet rich in coenzyme Q_{10} can slow down the decline in mitochondrial respiratory function normally observed in rat skeletal muscle (30). Myocardial reperfusion lowers the mitochondrial coenzyme Q_{10} content and stimulates the biosynthesis of coenzyme Q_{10} in young rats, but not in old rats (31). Dietary supplementation of coenzyme Q_{10} or the enrichment of cell membranes with monounsaturated fatty acids protects mitochondrial membranes of aged rats against attack by free radicals (32). The results presented here suggest the possibility of supplementation with exogenous coenzyme Q_{10} in old age, in particular in neuromuscular diseases. In post-poliomyelitis syndrome, it was found that the energy metabolism in skeletal muscle was positively influenced by oral supplementation with coenzyme Q_{10} (33). However, combined coenzyme Q_{10} and alpha-tocopherol acetate supplementation showed no antioxidative or membrane-stabilizing effect in young marathon runners (34,35).

In summary, our analyses of muscle biopsies from patients with neuromuscular diseases show that patients with specific diseases such as ALS or mitochondrial myopathies are particularly susceptible to oxidative stress. These patients might probably benefit from supplementation with antioxidants. The enzymatic antioxidative system is partly upregulated during aging, and the concentration of the lipophilic antioxidant coenzyme Q_{10} is reduced with increasing age. In some cases, the resulting overall antioxidative defense may not be sufficient to neutralize the increased oxidative stress shown in human skeletal muscle in old age, as is also suggested by other studies. A sufficient supply of antioxidants for aging healthily seems therefore advisable.

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Table 1: Characteristics of study patients with neuromuscular diseases

Diagnostic categories (number)	Gender male/female	Age (years) median (range)
Control group* (11)	5/6	55 (19 - 82)
Amyotrophic lateral sclerosis (sporadic form; 27)	14/13	67 (36 - 84)
Other motoneuron diseases (2)	2/-	56 (49 - 63)
Polyneuropathies (36)	23/13	60 (19 - 85)
Myositis (13)	4/9	59 (30 - 76)
Muscular dystrophy (11)	7/4	47 (26 - 77)
Mitochondrial myopathies (4)	2/2	64 (45 - 75)
Other metabolic myopathies (6)	2/4	57 (19 - 73)
Other myopathies (3)	2/1	56 (56 - 64)

*no neuromuscular disease diagnosed

Table 2: Antioxidative status and lipid peroxidation in muscle biopsies from patients with neuromuscular diseases

Diagnostic categories	Malondialdehyde [nmol/mg protein]	Glutathione peroxidase [U/mg protein]	Catalase [μmol/mg protein x min]	Superoxide dismutase [U/mg protein]	Alpha-Tocopherol [nmol/mg protein]	Coenzyme Q ₁₀ * [nmol/mg protein]
Control group	0.35 ± 0.04	2.0 ± 0.3	55.7 ± 6.8	3.1 ± 0.3	0.41 ± 0.08	0.29 ± 0.06 (33 ± 10)
ALS (sporadic form)	0.41 ± 0.04	2.4 ± 0.2	48.6 ± 3.5	3.1 ± 0.3	0.32 ± 0.04*	0.32 ± 0.03 (33 ± 5)
Other motoneuron diseases	0.90 / 0.50	n.m.	40.1 / n.m.	4.5 / n.m.	0.50 / 0.40	0.30 (9) / 0.20 (37)
Polyneuropathies	0.48 ± 0.04*	2.5 ± 0.2	46.4 ± 3.0	3.2 ± 0.3	0.44 ± 0.05	0.37 ± 0.04 (35 ± 4)
Myositis	0.36 ± 0.04	2.7 ± 0.4	51.9 ± 6.0	2.8 ± 0.4	0.53 ± 0.06	0.28 ± 0.04 (36 ± 7)
Muscular dystrophy	0.34 ± 0.07	2.0 ± 0.3	45.8 ± 4.3	2.0 ± 0.4	0.38 ± 0.05	0.29 ± 0.04 (40 ± 7)
Mitochondrial myopathies	0.64 ± 0.05*	2.4 ± 0.4	44.7 ± 17.9	4.2 ± 0.7	0.46 ± 0.19	0.34 ± 0.06 (33 ± 6)
Other metabolic myopathies	0.46 ± 0.11	2.0 ± 0.4	42.4 ± 9.4	2.6 ± 0.7	0.43 ± 0.08	0.44 ± 0.15 (24 ± 5)
Other myopathies	0.35 ± 0.04	2.2 ± 0.3	55.7 ± 6.8	1.6 ± 0.9	0.33 ± 0.14	0.27 ± 0.07 (13)

* shown in brackets are the percentages of reduced coenzyme Q₁₀ compared to total coenzyme Q₁₀; n.m. = not measured;

*In the stepwise multiple linear regression model the diagnostic categories were calculated as significant predictors (p<0.05); no diagnostic category was calculated as significant predictors for glutathione peroxidase, catalase, superoxide dismutase or coenzyme Q₁₀; Data are presented as mean ± S.E.M.

Figure. 1. Preparation of muscle biopsy samples for determination of antioxidative status and lipid peroxidation

Figure. 2. The activities of catalase (2A) and superoxide dismutase (2B) correlated positively with the age of patients and controls. The significant increase with age is shown for catalase ($R = 0.30$, $p < 0.01$) and superoxide dismutase ($R = 0.24$, $p < 0.01$), based on simple linear regression analysis (showing in addition the 95 % confidence bands of the true mean of y). The original statistical analysis were done with a multivariate procedure.

Figure. 3: The concentrations of coenzyme Q_{10} correlated negatively with the age of patients and controls. The significant decrease with age is shown ($R = -0.28$, $p = 0.0028$), based on simple linear regression analysis (showing in addition the 95 % confidence bands of the true mean of y). The original statistical analysis was done with a multivariate procedure.

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