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ELEVATION OF THE LEVEL OF THIOBARBITURIC ACID-REACTIVE PRODUCTS IN HINDLEG SKELETAL MUSCLE OF DYSTROPHIC MICE, BUT NON-ELEVATION IN TONGUE MUSCLE

K. WATANABE,* K. YAMADA,** T. MIZUTANI,** and T. TOTSUKA*

**Department of Physiology, Institute for Developmental Research, Aichi Prefectural Colony, Aichi 480-03 and **Faculty of Pharmaceutical Sciences, Nagoya City University, Nagoya 467 Japan*

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In order to understand the pathogenesis of mouse muscular dystrophy, we investigated the levels of the thiobarbituric acid-reactive substances (TBARS), H_2O_2 and NADPH oxidase activity, which were relative to the acceleration of oxidative conditions, in tongue and hindleg skeletal muscles from C57BL/6J-*dy* mice. The TBARS content (702 nmol/g protein) in skeletal muscles from 2-months-old dystrophic mice was increased significantly over that (384 nmol/g protein) in muscles from age-matched normal mice. The H_2O_2 concentration in dystrophic skeletal muscles was 30% higher than that in normal ones. Microsomal NADPH oxidase activity which was related to the production of superoxide anions, was similar between dystrophic muscles (4.66 nmol/10 min/mg protein) and normal muscles (4.11 nmol/10 min/mg protein). These results indicate that oxidation is accelerated in the dystrophic muscles. However, the TBARS content in the tongues of dystrophic mice was identical to that of normal mice. This finding supports our bone-muscle growth imbalance hypothesis for the pathogenesis of mouse muscular dystrophy.

KEY WORDS: Muscular dystrophy; Oxidative stress; Thiobarbituric acid-reactive substances; Malondialdehyde; Tongue; Dystrophic mouse.

Abbreviations used: TBARS, thiobarbituric acid-reactive substances; DHA, docosahexaenoic acid; GSHPx, glutathione peroxidase; SeCys, selenocysteine; HRP, horseradish peroxidase

INTRODUCTION

We are studying morphologically and biochemically *dy* mice (C57BL/6J *dy-dy*) to learn the etiology of progressive muscular dystrophy and to develop a treatment for the disease. Based on our previous findings, challenging the popular "muscle degeneration theory", we have proposed a "muscle defective maturation theory" and a further "bone-muscle growth imbalance hypothesis".[1,2,3] The *dy* mice appear to suffer from a defect in the mechanism of bone-growth-induced muscle growth. Stretched-contraction-induced damages followed by repair, instead of degeneration-regeneration in the muscle degeneration theory, would frequently occur locally along dystrophic muscle fibers during movement, causing overinflammation, espe-

Address for: Dr. Takaharu Mizutani, Faculty of Pharmaceutical Sciences, Nagoya City University, Tanabe-dori, Mizuho-ku, Nagoya Japan 467, Fax: (81) 52 834 9309

cially during rapid growth periods. This hypothesis thus implies a taboo (intensive exercise) and two possible symptomatic treatments, bone- or body-growth inhibitors and antiinflammatory drugs.

While *mdx* mice are believed to be deficient in dystrophin and to be the ideal animal model for Duchenne muscular dystrophy, it remains to be revealed what type of human dystrophies could be studied using *dy* mice. Strangely enough, however, the progressive course and severity of muscle abnormality and disability in patients with Duchenne dystrophy are well known to be much more similar to those in *dy* mice than in *mdx* mice. In this connection, it is noteworthy that histopathological changes are similar in various types of muscular dystrophies and even other muscle diseases, such as myositis. Our bone-muscle imbalance hypothesis may become a basis for considering the secondary progressive course of human muscle diseases and for the treatment of human muscle diseases.[4]

The findings that the amount of docosahexaenoic acid (DHA) in phospholipids in muscles of dystrophic mice was less than that of normal mice have been previously reported.[5,6] We confirmed these findings in skeletal muscles and also found that the amount in the tongues of dystrophic mice was identical to that of normal mice.[7] These results are consistent with our "bone-muscle imbalance hypothesis" that there is no pathological abnormality in the tongue muscles, whether normal or dystrophic, which is not influenced by bone growth. It is known that there is white muscle disease caused by selenium (Se) deficiency in animals.[8] We reported that the contents of Se-dependent glutathione peroxidase and selenocysteine (SeCys) tRNA in dystrophic muscles were increased,[9] and presented some properties of murine SeCys synthase.[10]

From these findings, we considered that the decrease in the DHA content was a result of removing the oxidized DHA from phospholipids by phospholipase A_2[11] in the elevated oxidative state in dystrophic muscles[12] and the increase of GSHPx activity should play to terminate the oxidative state. Meanwhile, it is well known that thiobarbituric acid-reactive substances (TBARS) are produced from DHA in the oxidative state. There were reports about the increase in TBARS content resulting from the decrease in DHA content in some dystrophic animals: humans,[13,14,15]; chicken,[16,17]; mice.[1,6] However, the finding that the TBARS content showed no difference in the dystrophic mice has also been reported recently.[18] In order to clarify this discrepancy of the TBARS level in murine dystrophic muscles, we determined the TBARS content in dystrophic muscles and tongue. We show an increase of TBARS in hindleg skeletal muscles and no difference in tongue muscles in this report. We also show the H_2O_2 content, which is closely related to intracellular peroxidation, and the activity of NADPH oxidase, which is related to superoxide anion formation.

MATERIALS AND METHODS

Animals

Dystrophic (*dy/dy*) and normal (?/+) mice of the strain C57BL/6J-*dy* were used. This strain originated from the Jackson Laboratory (Bar Harbor, ME, U.S.A.) and has been maintained by sib mating for over 50 generations in our laboratory.

Methods

The TBARS content was assayed by the method of Ohkawa.[19] The tissues were weighed and homogenized with 0.9% NaCl in a glass-Teflon homogenizer. Thirty percent TCA, 5N HCl and 0.75% sodium thiobarbiturate were added to the homogenate, and the mixture was heated at 95°C for 30 min. After cooling with tap water, the mixture was centrifuged at 3000 rpm for 10 min and the absorbance of the upper layer was measured at 535 nm. The TBARS content was normalized by protein concentration, because the protein content is the same level in normal and dystrophic muscles.[6] Protein concentration in homogenates was determined according to Ref.[20]

H_2O_2 determination in tissues was done by means of the loss of fluorescence of scopoletin (7-hydroxy-methoxy-coumarin) during its oxidation by horseradish peroxidase (HRP).[21] The tissues were weighed and homogenized with a mixture of 50 mM Tris-HCl (pH 7.5), 150 mM KCl, 10 mM $MgCl_2$, and 0.5 mM NaN_3. The homogenate was allowed to stand at 4°C for about 20 min prior to centrifugation for 10 min at 2°C, and after centrifugation at 22,000 g, 1.5 ml of the supernatant was transferred into test tubes and neutralized (pH 7.0) by the addition of 2 M triethanolamine buffer (pH 10.3) containing 0.15 M KOH with vigorous stirring. Aliquots were transferred into test tubes containing a mixture of 1 ml of 50 μM scopoletin and 0.1 ml of HRP (400 μg/ml). The mixture of sample, scopoletin, and HRP was stirred and incubated for 2 min at room temperature. The reaction was terminated by addition of 0.15 M tetraborate buffer. For blanks, Tris-HCl buffer (pH 7.0) was used instead of samples. Fluorescence was measured in a 204 fluorescence spectrophotometer (Hitachi; excitation at 350 nm, emission at 460 nm).

NADPH oxidase activity was measured in muscle homogenates prepared from murine muscles in 0.25 M sucrose-10 mM Tris-HCl buffer (pH 7.5).[22] The homogenate was centrifuged at 20,000 g for 20 min and the supernatant was further centrifuged at 105,000 g for 1 hr. The pellet was used as the microsomal NADPH oxidase preparation. NADPH oxidase activity was determined spectrophotometrically at 27°C. The decrease in absorbance at 340 nm was monitored with time and found to be linear for at least 6 to 10 min. The incubation system contained 0.1 M Tris-HCl buffer (pH 7.2), 0.012 mM $FeCl_3$, 0.15–0.20 mg protein and 4.0 mM ADP in a final volume of 1 ml. The reaction was initiated by the addition of 30 or 40 μl of a 3.0 mM solution of NADPH. The control cuvette contained all additions except NADPH.

RESULTS

Figure 1 shows the TBARS content of hindleg muscles of normal and dystrophic mice. The content of TBARS (702 ± 69 nmol/g protein) in dystrophic muscles at 2 months was increased significantly over that in age-matched normal muscles (384 ± 29 nmol/g protein). The symptom in the hindlimb of a dystrophic mouse was first manifested around 2 weeks of age. However, at 3 weeks, there was no significant difference between the TBARS levels in dystrophic and normal mice. These results suggest that the increase in TBARS content is not a primary event of dystrophy but a secondary pathological change. Figure 1 also showed that the difference in the TBARS content increased according to growth. The bodyweight of normal and dystrophic mice was already reported to be 23.3 ± 2.6 g and 15.6 ± 2.2 g

(mean ± SD) at 2 months, respectively.[23] Meanwhile, the rate of the increase (702 − 384 = 318 nmol/g protein) in TBARS content in dystrophic muscles was about 1% of that of the decrease (26 μmol/g protein) in fatty acid DHA content in dystrophic muscles.[7]

The TBARS content in the tongue in 4-week- and 4-month-old mice is shown in Figure 2. No significant difference was seen in the TBARS content between normal

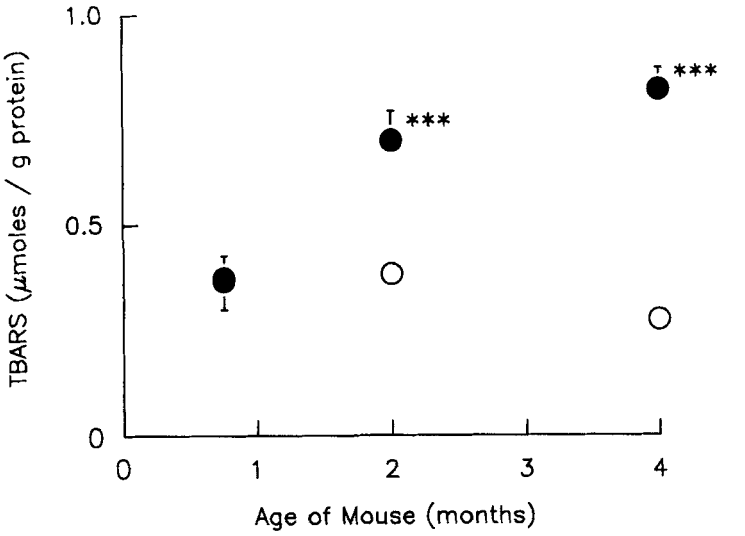

FIGURE 1 Thiobarbituric acid-reactive substance (TBARS) content of hindleg skeletal muscles of normal (○) and dystrophic (●) mice. The data represent the mean of 4 independent determinants. The points at 3 weeks are overlapped. Vertical bars represent the S.D. of the data and some bars overlap with in the circles. *** indicate a significant difference ($p < 0.001$).

FIGURE 2 TBARS content of tongue muscles of normal (○) and dystrophic (●) mice. The conditions are the same as those in Fig. 1.

and dystrophic tongues at 4 weeks and 4 months. These results support our hypothesis that dystrophy is caused by bone-muscle growth imbalance, because tongue muscles are voluntary striated muscles. The skeletal muscles which have pathological abnormalities in the dystrophic mice stick to bones and this causes mechanical stretching. However, this does not occur with the tongue, thus there is no mechanical stretching. This is the key difference between them. We have reported that there is no difference histochemically and biochemically between normal and dystrophic tongue muscles which are not influenced by bone growth.[24] This result for TBARS content in tongues, as well as that for DHA content,[7] may support our hypothesis.

The contents of TBARS in brain and liver also did not show a significant difference between the dystrophic and normal mice and no significant change in TBARS content with age has been seen, as shown in Figure 3. The content of TBARS in the brain was higher than in muscle and liver. We considered that elevation of the TBARS level and the decrease in DHA in dystrophic muscles is related to a disturbance of the oxidative conditions in cell cytosol. Therefore, we determined the H_2O_2 content. The H_2O_2 content in skeletal muscles was measured at 2 and 4.6 months of age (Table I). This shows a 29% and 62% increase in H_2O_2 content in dystrophic muscles as compared with normal muscles at 2 and 4.6 months, respectively.

The TBARS content was higher in dystrophic skeletal muscles, and this result

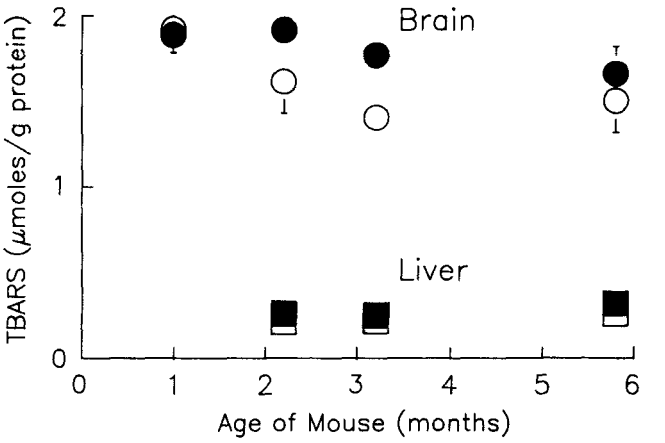

FIGURE 3 TBARS content of brain (circles) and liver (squares) of normal (open) and dystrophic (closed) mice. The conditions are the same as those in Fig. 1.

TABLE I
H_2O_2 contents in skeletal muscles of normal and dystrophic mice

Months	nmol/g wet weight[a] Normal	Dystrophic
2.0	17.3 ± 7.7	22.4 ± 7.2 (129%)[b]
4.6	19.3 ± 8.4	31.3 ± 6.2 (162%)

[a]Values are expressed as mean ± SD of 4 or 5 determinations.
[b]%of normal value.

might correlate with the significant decrease in DHA content in the hindleg muscles of dystrophic mice. Therefore, we measured the levels of NADPH oxidase activities O_2^- can attack DHA by peroxidation and produce TBARS, as shown schematically in Figure 4. NADPH oxidase is a membrane-bound enzyme, and we prepared the microsomal fraction by centrifugation (see MATERIALS AND METHODS) for measurement of the activities. The levels of microsomal NADPH oxidase rates in hindleg muscles of normal and dystrophic mice (3 months old) were 4.11 ± 1.70 and 4.66 ± 1.82 nmol/10 min/mg protein, respectively. No significant difference was shown in the activities of microsomal NADPH oxidase between normal and dystrophic muscles, although the activity in dystrophic muscles was slightly higher than that in normal muscles.

DISCUSSION

As shown in the scheme in Figure 4, the polyunsaturated fatty acid DHA is oxidized by the superoxide anion O_2^-, decreasing the content of DHA.[7] This results in a doubling of the TBARS level. The difference in the activity of NADPH oxidase was insignificant between normal and dystrophic muscles. Meanwhile, it has recently been reported that the number of mitochondria and some enzyme activities, such as NADH and succinate dehydrogenases, in mitochondria are much higher in dystrophic skeletal muscles than in normal muscles.[24,25,26] Therefore, we considered that some of superoxide anion O_2^- might have leaked from mitochondria, in which the anions are produced by an electron-transport system, into the cytosol of skeletal muscles.

Some reports showed that the superoxide dismutase (SOD) activity was generally lower (−44%) in dystrophic mice,[27,28] and this is a partial cause of the accumulation of O_2^-. The tendency to increase the oxidative level was found in the H_2O_2 content (30%) of dystrophic muscles. The activity of GSHPx increased and SeCys tRNA, which is used for the cotranslational incorporation of SeCys into GSHPx, also increased.[9] These increases may play a role in compensating for the increase in H_2O_2. These increases might be regulated on the transcriptional level of these

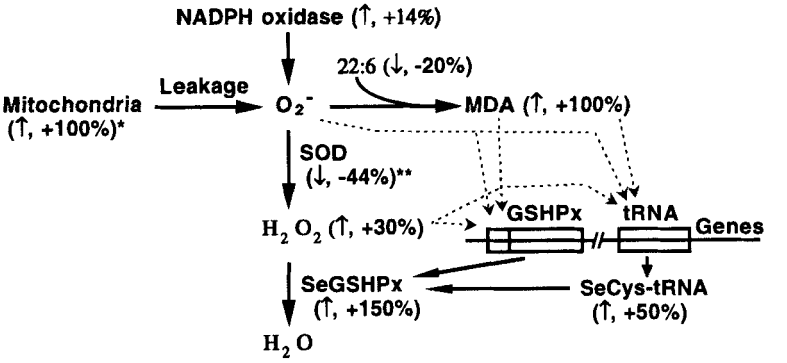

FIGURE 4 Scheme for the acceleration of the oxidative state in dystrophic skeletal muscles. The % changes of dystrophic to normal muscles are shown in parentheses. MDA is malondialdehyde, produced from DHA (22:6). MDA is one of major TBARS. In the figure, * and ** are the results obtained from Silverman[25] and Burr[27], respectively.

genes of GSHPx and tRNA by some inducers, such as superoxide anions, TBARS or H_2O_2.

Although the primary cause for the elevation of oxidation in dystrophic muscles is still obscure, the elevation of oxidation, such as the increase in NADPH oxidase activity, the decreased SOD activity, the increased enzyme activities for O_2^- production in mitochondria and the high H_2O_2 level are noteworthy in dystrophic muscles. These elevations introduced the decrease in the DHA level and the increase in the TBARS level. However, there is no evidence of a cause-effect relationship between changes in superoxide, hydrogen peroxide, TBARS, and a loss of DHA in dystrophic muscles. In general, we can conclude that an accelerated state of oxidation is maintained in dystrophic muscles. This high oxidation level may not be the primary pathological event but rather a secondary one. This high level must be a result of inflammation in dystrophic muscles and we propose that the primary change of dystrophy is due to the bone-muscle growth imbalance, because we could not find any oxidative change in tongue muscles, as reported in this paper.

Finally, in this report, we show the increase in TBARS content in dystrophic skeletal muscles. This result coincides with some reports on humans,[13,14,15] chicken,[16,17] and mice,[17] but not with the results for mice obtained by Asayama *et al.*[18].

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