β-receptors are found. The next question is whether the vesicles observed in the low sucrose region of the gradient are similar to the light density vesicles described by others for β-receptors. After pre-incubation of intact cells with tritiated NMS, which does not cross the plasma membrane[16], followed by gradient centrifugation, a distribution pattern is found which is identical to that found when the gradient fractions are incubated with either QNB or NMS. Furthermore, upon post-incubating the fractions of the gradient obtained with pre-incubated cells in monolayers, no further increase of bound radioactivity was found. This means that all binding sites are already occupied during the first incubation. If the low-density fractions in the unstimulated cells were to consist of intracellular vesicular binding sites, NMS would not have labeled these binding sites and, consequently, post-incubation would result in an increase of the bound radioactivity in this region of the gradient. That this is not the case suggests that the QNB binding site in the low sucrose region of the gradient is not associated with an intracellular particle, but present at the outer surface of the plasma membrane.

We conclude that at least two different QNB binding sites are present in N1E 115 mouse neuroblastoma cells. These forms are separated by non-linear gradient centrifugation methods. We suggest that the binding site in the low sucrose region of the gradient is localized in a vesicle of membranous origin but not by a process of sequestration. These vesicles could originate by a process called 'vesiculization' or 'membrane shedding'. This process, described by Scott[17,18], results in extracellular particles. They contain part of the membrane and membranous components and are shed by the cells through an energy-dependent mechanism. Transformed cells are extremely active in this respect, non-transformed cells much less so. Further experimentation will be needed to establish the exact nature of the low-density ligand binding.

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Increased susceptibility to lipid peroxidation in skeletal muscles of dystrophic hamsters

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Summary. The results showed that the total content of lipids, which could be peroxidized with Fe(2 +)/ascorbate stimulation in vitro, was 45.4% and 53.7% higher than normal in the dystrophic hamster muscle at the age of 1 and 3 months, respectively. Correspondingly, the susceptibility to lipid peroxidation (stimulated by ADP-chelated iron at 37 °C) was 38.6–74.3% higher in dystrophic muscles. The increases were not related to necrotic lesions and inflammation observed. The activities of glucose-6-phosphate dehydrogenase, glutathione reductase, thioredoxin reductase and catalase were increased in dystrophic muscles but those of superoxide dismutases and glutathione peroxidase were unaffected.
Key words. Lipid peroxides; antioxidants; muscular dystrophies; skeletal muscles; hamster.

The mechanism of cellular damage in genetic muscular dystrophies is unknown, although human cDNA and the protein product, dystrophin, of the Duchenne muscular X-linked locus have recently been identified[1]. Dystrophin is localized in the sarcolemma[2–4] and t-tubular membranes[3] of muscle fibers. One possible cause for

membrane injuries, primary or secondary, could be a free radical-induced peroxidation of polyunsaturated lipids in muscle membranes. Different lines of evidence suggest that in dystrophic muscles disturbances occur in the balance between free radicals and their scavengers. An increased level of malondialdehyde, a product of lipid peroxidation, has been recorded in dystrophic muscles[5-8], as well as an increased level of certain antioxidative enzymes[6-9], probably as an adaptation to an increased formation of free radicals and of lipid peroxides.

The purpose of this study was to measure, whether the muscles of dystrophic hamsters are more susceptible to increased oxidative stress in vitro, or whether the level of peroxidizable lipids is increased. The activities of the main scavenger enzymes, directed against oxidative stress, were also recorded.

Materials and methods

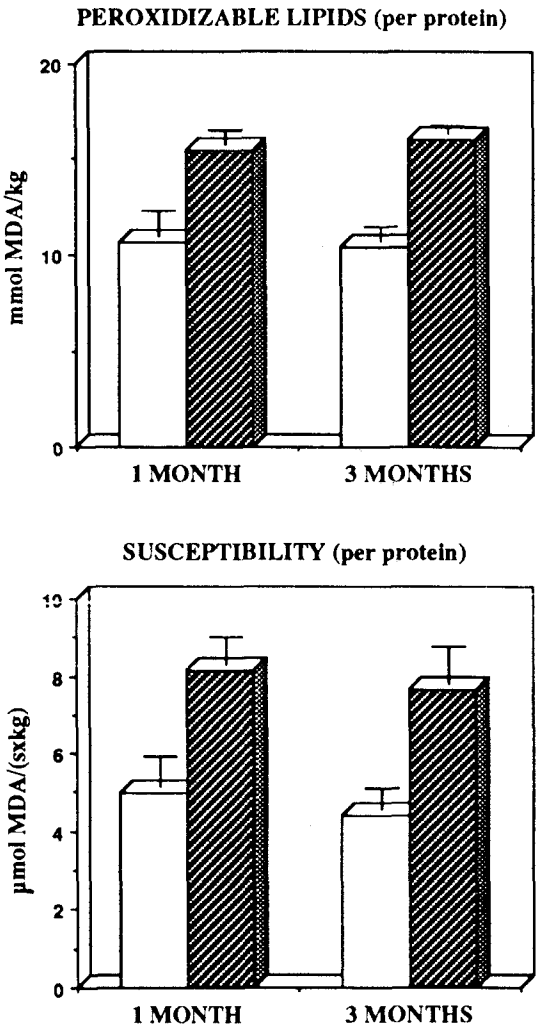
This study was conducted with male BIO 14.6 hamsters (Biobreeders, Watertown, Massachusetts). Animal care essentially was as earlier[10,11]. To characterize prenecrotic changes, the age groups of 1 and 3 months were elected. Quadriceps femoris muscles were studied from 8 controls (for both ages) and 8 and 7 (1 and 3 months) dystrophic hamsters with both biochemical assays and histochemical stainings. The weights of hamsters for controls and dystrophics were 72 ± 2 ($\pm$ SE) and 65 ± 3 (1 month) (statistically nonsignificant) and 152 ± 3 and 90 ± 5 g (3 months) ($p < 0.001$, ANOVA).

The total content of peroxidizable lipids was assayed in muscle homogenates, using Fe(2 +)/ascorbate-stimulation in ice-bath and carrying the reaction to completion, as described in our earlier papers[11-13]. This procedure excludes enzymatic antioxidants. The susceptibility to lipid peroxidation in vitro was measured by assaying NADPH-dependent (ADP-chelated and iron-induced) lipid peroxidation at 37 °C as described earlier in detail[12]. Lipid peroxidation was measured in both assays as malonialdehyde production[12]. The activity of catalase, and the contents of total sulfhydryl groups and those of carnosine and anserine, were assayed in the homogenate. The activities of superoxide dismutases (both Cu, Zn-SOD and Mn-SOD), glutathione peroxidase (Se-dependent), glutathione reductase, thioredoxin reductase, glucose-6-phosphate dehydrogenase and creatine kinase were assayed in a supernatant fraction ($590 \times g$, 10 min). The assay methods were described in our earlier papers[10,11,13], and those for glutathione and thioredoxin reductases[15] and carnosine and anserine[16] elsewhere.

Transverse cryostate sections were cut from the proximal head of the quadriceps femoris muscle at various depths, and stained with hematoxylin and eosin to evaluate pathologic changes. Some samples were also cut from tibialis anterior and gastrocnemius muscles.

Results and discussion

The total content of peroxidizable lipids, inducible by a strong Fe(2 +)/ascorbate-stimulation, was considerably higher in dystrophic muscles already at the age of one month (fig.). The difference was statistically significant when compared to wet weight or protein content, which was lower in dystrophic muscles (table). The difference in the content of peroxidizable lipids is not due to necrotic lesions and inflammation, because the occurrence of necrotic fibers was very infrequent at the age of one month, and because the difference remained the same in the older age group, where necrotic lesions occurred frequently. Our earlier observations of strenuous, exercise-induced, skeletal muscle fiber injuries[13] showed, that the level of necrotic lesions and the occurrence of inflammatory cells does not affect the content of peroxidizable lipids in skeletal muscles. The difference in the content of lipids, susceptible to peroxidation in vitro, suggests changes in



Total content of peroxidizable lipids and susceptibility to lipid peroxidation in muscle homogenates of normal (□) and dystrophic (■) hamsters. Values are means $\pm$ SE. Statistical significances (ANOVA): $p < 0.01$ for all per protein-values and $p < 0.05$ for all per wet weight-values. Abbr.: MDA, malondialdehyde.

Activities of enzymatic antioxidants in normal control and dystrophic hamster skeletal muscle at the ages of 1 and 3 months.

Variable	1 month Control	Dystrophic	3 months Control	Dystrophic
Superoxide dismutase (SOD)				
Cu, Zn-SOD	79.1 ± 7.2	52.5 ± 9.2	89.2 ± 11.5	68.6 ± 10.9
Mn-SOD	95.4 ± 8.5	106.8 ± 7.5	109.3 ± 8.8	114.4 ± 6.8
Catalase	7.3 ± 0.5	12.9 ± 1.0***	8.7 ± 1.0	13.1 ± 1.9*
Glutathione peroxidase	36.2 ± 4.5	47.8 ± 6.3	50.3 ± 7.5	63.6 ± 7.4
Glutathione reductase	140 ± 2	267 ± 5***	na	na
Thioredoxin reductase	93 ± 3	116 ± 3***	na	na
Glucose-6-P dehydrogenase	255 ± 23	685 ± 104***	177 ± 15	708 ± 185***
Creatine kinase	4.86 ± 0.24	3.10 ± 0.15***	na	na
Protein content	177 ± 7	150 ± 4**	181 ± 4	161 ± 5**

Values are means ± SE. Enzyme activities are given as μkat/kg protein for glutathione peroxidase, glutathione reductase, thioredoxin reductase and glycose-6-P dehydrogenase, kat/kg protein for creatine kinase, U/mg protein for superoxide dismutases, and U/mg protein for catalase[13]. Protein contents are g/kg wet weight. Abbr.: na, not assayed. Statistical significances (ANOVA): *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

the physicochemical state of membrane lipids, perhaps due to a deficiency in stabilizing protein. The changes in the peroxidative components of sarcolemmal and t-tubular systems, where dystrophin has been localized[2-4], should be studied in the future, although problems occur in the purification of skeletal muscle membrane fractions. However, earlier studies[17] have shown increased phospholipid and cholesterol contents in sarcolemma and t-tubular membranes in dystrophic chickens.

Another way to evaluate lipid peroxidation capacities in vitro is to measure the speed of lipid peroxidation in homogenates exposed to oxidative stress. This can be measured e.g. from Fe(2+)/ascorbate-catalyzed reaction[11,14] or more reliably from NADPH-dependent, ADP-chelated, iron-induced, lipid peroxidation at 37 °C, when the cellular defence mechanisms are active. The susceptibility assays showed that the skeletal muscle homogenates of dystrophic muscles were 38.6–74.3 % more susceptible to induced lipid peroxidation than those of normal muscles (fig.). Most probably this reflects the difference in substrate pool, observed as an increased level of peroxidizable lipids, rather than the deficiency in some antioxidant.

Several studies[6-9] have shown increased activities of enzymatic antioxidants in dystrophic muscles. The degree of these increases has varied, perhaps due to the type of dystrophy (human/ animal models), the phase of dystrophy, and the muscles studied. In the present study the most prominent changes occurred in the activities of glutathione reductase, glucose-6-phosphate dehydrogenase and catalase, whereas the activities of superoxide dismutases and glutathione peroxidase were unaffected (table). The strong increase in the activity of glucose-6-phosphate dehydrogenase reflects a stimulation of the pentose phosphate pathway and is a typical change in damaged and diseased muscles[10].

Central nuclei in muscle fibers without necrotic lesions were frequent at the age of one month, whereas scattered necrotic fiber injuries and inflammation occurred at the age of three months. The lower activity of creatine kinase in dystrophic muscle at the age of one month could be due to the inherently increased permeability of sarcolemma. The simultaneous increases in the activities of glucose-6-phosphate dehydrogenase and glutathione reductase potentiate the function of the glutathione redox cycle as the scavenging of lipid peroxides by glutathione peroxidase, although the activity of glutathione peroxidase was unchanged. The total contents of sulfhydryl groups and anserine and carnosine were unaffected (data not shown). In conclusion, it seems that the lipid peroxidation capacity is increased in dystrophic hamster muscles. Factors inducing free radical formation, e.g. vitamin E and selenium deficiencies, as well as the activation of neutrophils and macrophages in dystrophic muscles may be destructive for surviving muscle fibers.

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