Oxidation state of tissue thiol groups and content of protein carbonyl groups in chickens with inherited muscular dystrophy

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Indirect evidence suggests that oxidative stress may play a role in the pathogenesis of inherited muscular dystrophy, but the significance and precise extent of this contribution is poorly understood. Compared with normal muscle, significantly higher contents of glutathione, glutathione disulphide, protein–glutathione mixed disulphides and protein carbonyl groups, and significantly lower contents of free protein thiol groups, were found in pectoralis major muscle of genetically dystrophic chickens (the muscle affected by this disease) at 4 weeks of age. Other tissues did not show such marked disease-related differences. Interestingly, the protein pool in normal, but not dystrophic, pectoralis major muscle was relatively less oxidized in relation to the glutathione pool as compared with other tissues studied. The mechanisms by which this unique relationship between the thiol pools is maintained remain unknown. Although the physiological consequences of the increased content of protein carbonyl groups and the altered thiol pools in dystrophic muscle are not clear, the changes evident at such a young age are consistent with the occurrence of oxidative stress and may reflect significant damage to cellular proteins in this disease.

INTRODUCTION

Glutathione (GSH), a tripeptide (γ -glutamylcysteinylglycine) present in high concentrations in the cytoplasm of cells, can act directly as a soluble antioxidant, but its main function is as a co-substrate in various cellular enzymic reactions in which it is oxidized to glutathione disulphide (GSSG) or forms stable covalent adducts with various electrophiles [1–3]. Cysteinyl residues, which are often critical to the maintenance of protein structure and enzymic activity, can be disrupted via the oxidation of the free thiol groups. A tissue's content of GSH relative to GSSG is believed to maintain indirectly the reduced state of protein thiol groups. Carbonyl groups, which are normally absent from cellular proteins, are thought to be formed after free-radical attack on amino acid residues. Thus the formation of protein carbonyl groups, the loss or oxidation of protein thiol groups and the oxidation of GSH to GSSG may all be physiologically relevant estimates of oxidative stress [1,4].

Many differences between the physiology of normal and dystrophic muscle have been noted, but the consensus regarding these alterations is that they represent secondary responses of muscle tissue to some as yet unknown primary defect [5]. The product of the Duchenne-muscular-dystrophy locus has been identified as a 400 kDa protein known as dystrophin [6], whose levels inversely correlate with the severity of the disease [7]. The function of this protein remains unknown. Omaye & Tappel [8] were the first to suggest that oxidative stress plays a role in the pathogenesis of muscular dystrophy. The consistent finding of elevated activities of anti-oxidant enzymes and of products of lipid peroxidation in the muscle of dystrophic chickens may reflect a higher degree of continuing oxidative stress in this tissue

[5,8–10]. The broad similarity between the pathophysiology of muscular dystrophy and oxidative damage in normal muscle also supports this idea [11].

Evidence regarding the source, extent and consequences of any oxidative stress in dystrophic muscle is incomplete and has often involved tissue obtained at late stages of the disease. In the present study, the content and oxidation state of various thiol pools and the carbonyl-group content of proteins in the chicken model of muscular dystrophy were analysed in both affected and unaffected tissues at stages of the disease (1–4 weeks of age) that precede cellular necrosis and macrophage infiltration [9]. The finding that thiol groups and proteins were oxidized in young dystrophic pectoralis major muscle tissue may indicate that this process plays a role in the altered protein metabolism and decreased enzymic activities noted in this disease.

MATERIALS AND METHODS

Materials

4,4'-Bis(dimethylamino)benzhydrol, DNA (from calf thymus, type I), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), *N*-ethylmaleimide, α -D(+)-glucose (grade III), D-glucose 6-phosphate (disodium salt, dihydrate), glucose-6-phosphate dehydrogenase (type XXIII, from *Leuconostoc mesenteroides*), GSH (free acid), GSSG (grade III, free acid), glutathione reductase (EC 1.6.4.2, type III, from baker's yeast), guanidine hydrochloride, NADP⁺ (monosodium salt, from yeast β -NAD) and NADPH (tetrasodium salt, type I) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. 2,4-Dinitrophenylhydrazine (DNPH, moist solid with 30% water), 2,2'-dipyridyl (99+ % pure) and dithiothreitol (99 %

Abbreviations used: DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); DNPH, 2,4-dinitrophenylhydrazine.

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pure) were obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Bio-beads (SM-2, 20–50 mesh) were obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. N_2 gas (pre-purified) was obtained from Big Three Industries, LaPorte, TX, U.S.A. All other chemicals used were of reagent or spectrophotometric grade.

Animals

Normal white Leghorn (strain S10) and dystrophic (strain S11) chickens were obtained as eggs from the Department of Animal Genetics at the University of Connecticut, Storrs, CT, U.S.A. Chicks were raised with free access to Purina Chick Starter Chow and water. The room was maintained at 25 °C with a 12 h light–dark cycle. Food was removed for 16 h before chickens were killed by cervical dislocation, and tissues were removed for analysis.

Protein carbonyl-group assay

Pectoralis major and soleus muscles were removed from 1-, 2- and 4-week-old chickens and rapidly frozen in liquid N_2 . The heart and liver were then perfused with ice-cold phosphate-buffered saline (130 mM-NaCl/50 mM- KH_2PO_4 , pH 7.4), removed and similarly frozen. Frozen tissues were powdered with a mortar and pestle (pre-cooled with liquid N_2) and stored under N_2 at –80 °C until analysed.

Protein carbonyl groups were determined by the technique of Oliver *et al.* [12]. Tissue powder (50–100 mg) was homogenized in 4 ml of ice-cold 50 mM- KH_2PO_4 , pH 7.4, containing 5 mM-EDTA. Duplicate 1 ml portions of the homogenate were precipitated with 1 ml of 20% (w/v) trichloroacetic acid. After centrifugation at 10000 *g* for 15 min, the pellets were suspended by gentle homogenization in 2 M-HCl with and without 0.1% DNPH. Both samples were incubated with shaking at room temperature for 1 h, then the proteins were reprecipitated with 1 ml of 20% trichloroacetic acid and centrifuged as above. The precipitates were washed three times with ethanol/ethyl acetate (1:1, v/v) to remove lipids and DNPH that had not reacted. The final precipitate was suspended with gentle homogenization in 2 ml of 6 M-guanidine/HCl, heated briefly on a boiling-water bath and centrifuged to remove insoluble material. Carbonyl groups were estimated by measuring the A_{370} of the DNPH derivatives in the suspension and subtracting the A_{370} of the non-modified protein. Absorption spectra (not shown) were plotted for all tissues from both the normal and dystrophic strains to verify the presence of the DNPH derivative. Exogenous DNA was added to other samples to verify the lack of interference by excess DNA under these conditions. The final data were expressed as nmol of carbonyl groups/mg of protein by using a molar absorption coefficient of 21 000 litre·mol⁻¹·cm⁻¹ for the DNPH derivatives [12].

Assays of thiol pools

Compared with values obtained on tissue analysed immediately after freeze-clamping, the storage of tissue powders sealed under N_2 at –70 °C for up to 4 weeks did not lead to measurable losses of GSH or protein thiol groups, nor to increases in GSSG or protein–GSH mixed disulphides. GSH, GSSG, acid-soluble thiols, protein–GSH mixed disulphides and total reduced protein thiol groups were measured by the general procedures recom-

mended by Lou *et al.* [13]. Tissue powder (100–200 mg) was homogenized in 4 ml of ice-cold 0.3 M- $HClO_4$ containing 5 mM-EDTA, 0.06% bipyridine and 10 μ l of octanol. After centrifugation at 10000 *g* for 10 min, total acid-soluble thiols were measured by adding 1 ml of the neutralized supernatant to 1 ml of 2 mM-DTNB in 0.05 M- KH_2PO_4 /5 mM-EDTA, pH 7.4. The A_{412} was measured after 15 min. Results are expressed as nmol of total thiols (as GSH equivalents)/mg wet wt.

Total GSH + GSSG was measured in the neutralized supernatant sample by mixing 10–20 μ l with 0.7 ml of buffer (0.1 M- NaH_2PO_4 /5 mM-EDTA, pH 7.4), followed by 100 μ l of 6 mM-DTNB and 10 μ g of glutathione reductase [14]. The reaction was initiated with 100 μ l of 2 mM-NADPH, and the change in A_{412} was measured over 2 min.

Protein thiol groups were measured in the protein pellet that had been washed once with 3 ml of 0.3 M- $HClO_4$ containing 5 mM-EDTA and 0.06% bipyridine, then once with ethanol/ethyl acetate (1:1, v/v) [15]. The pellet was dried under vacuum and suspended in 2 ml of 6 M-guanidine hydrochloride, mixed with 1 ml of 0.15 M- KH_2PO_4 /5 mM-EDTA, pH 7.4, and centrifuged at 10000 *g* for 10 min. A 50–200 μ l sample of the protein solution was mixed in a test tube with 0.8 ml of 0.05 M- KH_2PO_4 /5 mM-EDTA, pH 7.4, and 1 ml of 2 mM-DTNB. A replicate sample was mixed with 0.3 ml of buffer plus 0.5 ml of 10 mM-*N*-ethylmaleimide. After 5 min, 1 ml of the DTNB solution was added and the A_{412} of all samples was read after 30 min. Data are expressed as nmol of thiol groups (as GSH equivalents)/mg of protein, corrected for the background absorbance measured in samples treated with *N*-ethylmaleimide.

GSSG and protein–GSH mixed disulphides were determined in 100–200 mg of tissue powder homogenized in 4 ml of ice-cold benzhydrol reagent [0.06% 2,2'-bipyridine, 5 mM-EDTA, 0.5% 4,4'-bis(dimethylamino)-benzhydrol in 30% (v/v) acetic acid purged with N_2]. This reagent binds to and prevents oxidation of free thiol groups. Then 0.6 ml of 3 M- $HClO_4$ was added and the sample again homogenized. After centrifugation at 10000 *g* for 10 min, a 3 ml sample was removed and the pH adjusted to 4.5. This mixture was centrifuged at 10000 *g* for 10 min, and 2 ml of the resulting supernatant was added to 0.5 g of Bio-beads (to adsorb benzhydrol that had not reacted) and shaken slowly at room temperature for 30–40 min. Then 1 ml of the resulting clear solution was removed, neutralized, and assayed for GSSG by the enzymic procedure described above. Analyses of standard amounts of GSH run through the entire assay procedure revealed no evidence of spurious oxidation.

Protein–GSH mixed disulphides were determined in the protein pellet that had been washed with 4 × 3 ml of 0.3 M- $HClO_4$ containing 5 mM-EDTA and 0.06% bipyridine and then with 2 × 3 ml of methanol/diethyl ether (1:5, v/v). After centrifugation at 10000 *g* for 10 min, the pellet was dried under vacuum, and resuspended by gentle homogenization in 1.5 ml of reaction solution (0.5 mM-dithiothreitol, 0.2 mM-NADP⁺, 2.5 mM-glucose 6-phosphate in 25 mM- $Na_4P_2O_7$ /0.1 M-glycine/5 mM-EDTA, pH 8.4). A 50 μ l sample of the suspension was removed, mixed with 450 μ l of 6 M-guanidine hydrochloride, heated, then analysed for protein. The remaining suspension was mixed with 60 μ l of the enzyme solution (100 μ g of glutathione reductase/ml and 50 μ g

of glucose-6-phosphate dehydrogenase/ml in 0.1 M- NaH_2PO_4 /5 mM-EDTA, pH 7.4) and incubated at 37 °C for 30 min with shaking. The reaction suspension was then centrifuged at 10000 *g* for 10 min, and 1 ml of the supernatant was removed, heated in a boiling-water bath for 10 min to denature glutathione reductase, then cooled on ice. A portion of this final solution was assayed for GSH as described above.

General procedures

Protein was determined by modifications of the procedures of Bradford [16]. The A_{595} was measured for samples, blanks and known standards of bovine serum albumin. Statistical comparisons between groups were made by the two-tailed Student's *t* test, with a significance level of 0.05. Comparisons among multiple groups were made by an ANOVA (analysis of variance) program with Student–Newman–Keuls *post-hoc* comparisons [17]. Some analyses were made by a multivariate ANOVA program of the SAS statistical package.

RESULTS

The acid-precipitable proteins of dystrophic pectoralis major muscle had significantly higher than normal contents of carbonyl groups at 1, 2 and 4 weeks of age (Fig. 1). The carbonyl content of soleus muscle proteins was higher in samples from dystrophic birds at 2 weeks of age but was similar in the two strains at other ages. The carbonyl content of proteins from heart and liver tissue did not differ between normal and dystrophic strains (Fig. 1).

The GSH content of dystrophic pectoralis major

muscle was higher than normal at every age studied, reaching twice the normal values at 4 weeks of age (Table 1). There was, however, an age-dependent decrease in GSH in normal, and to a lesser extent in dystrophic, pectoralis major muscle. The GSH content of liver, but not heart, at 4 weeks of age was significantly higher than normal in dystrophic birds. The GSH contents of normal and dystrophic soleus muscle did not differ from one another, although there was a slight trend towards a decrease in GSH over time (Table 1). The content of GSSG was significantly higher than normal only in pectoralis major muscle of 4-week-old dystrophic birds (Table 1). Since both GSH and GSSG were increased, the ratio of GSH to GSSG was not significantly different between strains in any tissue at any age (Table 1).

The content of thiol groups in the proteins of dystrophic pectoralis major muscle were significantly lower than normal at 4, but not at 1 or 2, weeks of age (Table 2). An identical pattern was seen in soleus muscle, whereas the thiol content of proteins from liver and heart did not differ between normal and dystrophic strains at any age. Table 2 also shows the contents of protein–GSH mixed disulphides in proteins extracted from various tissues of normal and dystrophic birds. The proteins of dystrophic pectoralis major muscle had significantly higher contents of these mixed disulphides at 1 and 4 weeks of age. Since these proteins contained fewer free thiol groups, the ratio of free thiol groups to mixed disulphides was also significantly different between the two strains (Table 2). The proteins of soleus muscle, heart and liver from 4-week-old birds showed no differences in protein–GSH mixed disulphides content between normal and dystrophic strains (Table 2).

Fig. 1. Carbonyl-group content of proteins in various tissues of normal and dystrophic chickens

Carbonyl-group contents were measured in proteins isolated from various chicken tissues by their reaction with DNPH as described in the Materials and methods section, and are expressed as nmol of carbonyl residues/mg of protein (mean $\pm$ S.E.M.; $n = 3-4$): * significantly different from normal birds of the same age ($P < 0.05$). ND, not determined. ▨, Normal; ■, dystrophic.

Table 1. Contents and ratios of GSH and GSSG in normal and dystrophic chickens

GSH contents in the acid-soluble fraction of tissue extracts were determined by an enzymic recycling assay [14], modified as described in the Materials and methods section. Data are expressed as nmol/mg wet wt. (means $\pm$ S.E.M.; $n = 3-4$ for all data): * significantly different from normals at the same age ($P < 0.05$).

Tissue	Age (weeks)	GSH		GSSG		GSH/GSSG	
		Normal	Dystrophic	Normal	Dystrophic	Normal	Dystrophic
Pectoralis major	1	1.15 $\pm$ 0.05	1.52 $\pm$ 0.06*	0.011 $\pm$ 0.001	0.017 $\pm$ 0.003	105 $\pm$ 23	89 $\pm$ 8
	2	0.80 $\pm$ 0.04	0.95 $\pm$ 0.04*	0.011 $\pm$ 0.001	0.010 $\pm$ 0.001	73 $\pm$ 5	95 $\pm$ 19
	4	0.50 $\pm$ 0.04	0.98 $\pm$ 0.05*	0.017 $\pm$ 0.002	0.024 $\pm$ 0.003*	31 $\pm$ 4	43 $\pm$ 5
Soleus	1	1.22 $\pm$ 0.09	1.39 $\pm$ 0.03	0.019 $\pm$ 0.005	0.039 $\pm$ 0.009	64 $\pm$ 16	36 $\pm$ 8
	2	1.32 $\pm$ 0.04	1.22 $\pm$ 0.09	0.028 $\pm$ 0.006	0.040 $\pm$ 0.010	47 $\pm$ 9	31 $\pm$ 8
	4	1.00 $\pm$ 0.08	1.03 $\pm$ 0.02	0.025 $\pm$ 0.002	0.029 $\pm$ 0.002	40 $\pm$ 2	35 $\pm$ 3
Heart	4	2.04 $\pm$ 0.07	1.98 $\pm$ 0.09	0.068 $\pm$ 0.007	0.059 $\pm$ 0.011	30 $\pm$ 3	34 $\pm$ 7
Liver	4	1.59 $\pm$ 0.10	2.06 $\pm$ 0.08*	0.042 $\pm$ 0.003	0.049 $\pm$ 0.002	38 $\pm$ 5	42 $\pm$ 2

Table 2. Content and ratio of protein thiols and protein-GSH mixed disulphides in tissues of normal and dystrophic chickens

Protein thiols were determined by their reaction with DTNB, and protein-GSH mixed disulphides by measurement of GSH released from isolated tissue protein, by using procedures detailed in the Materials and methods section [12-14]. Data are expressed as nmol GSH equivalents or released GSH/mg of protein (means $\pm$ S.E.M.; $n = 3-4$ for all data): * significantly different from normals at the same age ($P < 0.05$).

Tissue	Age (weeks)	Protein thiols (A)		Protein-GSH mixed disulphides (B)		$10^{-3} \times A/B$	
		Normal	Dystrophic	Normal	Dystrophic	Normal	Dystrophic
Pectoralis major	1	42.9 $\pm$ 1.5	39.8 $\pm$ 1.3	0.021 $\pm$ 0.002	0.030 $\pm$ 0.002*	2.0 $\pm$ 0.3	1.3 $\pm$ 0.1*
	2	46.2 $\pm$ 0.8	43.5 $\pm$ 1.8	0.008 $\pm$ 0.001	0.010 $\pm$ 0.001	5.9 $\pm$ 0.7	4.5 $\pm$ 0.8
	4	54.1 $\pm$ 1.1	47.5 $\pm$ 1.8*	0.009 $\pm$ 0.001	0.019 $\pm$ 0.002*	6.0 $\pm$ 0.6	2.6 $\pm$ 0.3*
Soleus	1	33.8 $\pm$ 1.8	36.3 $\pm$ 0.7	0.040 $\pm$ 0.004	0.058 $\pm$ 0.009	0.8 $\pm$ 0.2	0.6 $\pm$ 0.1
	2	40.7 $\pm$ 2.2	39.6 $\pm$ 2.1	0.022 $\pm$ 0.002	0.041 $\pm$ 0.010	1.8 $\pm$ 0.2	1.0 $\pm$ 0.2
	4	43.8 $\pm$ 0.3	41.9 $\pm$ 0.4*	0.023 $\pm$ 0.002	0.028 $\pm$ 0.002	1.9 $\pm$ 0.2	1.5 $\pm$ 0.1
Heart	4	59.9 $\pm$ 2.2	57.5 $\pm$ 1.3	0.058 $\pm$ 0.007	0.041 $\pm$ 0.008	1.0 $\pm$ 0.1	1.4 $\pm$ 0.3
Liver	4	71.7 $\pm$ 1.9	72.5 $\pm$ 2.3	0.022 $\pm$ 0.001	0.023 $\pm$ 0.001	3.3 $\pm$ 0.3	3.2 $\pm$ 0.2

The relationship between the protein thiol/protein-GSH mixed-disulphide ratio and the GSH/GSSG ratio at 4 weeks of age is presented in Fig. 2. There was a significant difference in this relationship between normal and dystrophic strains only in pectoralis major muscle. Interestingly, the difference was mainly due to a greater state of protein thiol reduction relative to GSH content in normal pectoralis major muscle compared with all other tissues, either normal or dystrophic. This difference between normal and dystrophic pectoralis major was also evident, albeit to a lesser extent, at 2 weeks of age (ratios of 81 ± 8 versus 49 ± 8), but not at 1 week of age (20 ± 3 versus 15 ± 2). Heart tissue from dystrophic birds had a significantly different ratio between these parameters at 4 weeks of age, but it was small and in the opposite direction compared with the difference in pectoralis major muscle (Fig. 2). The ratio did not differ significantly between normal and dystrophic strains at earlier ages in soleus muscle (1 week 13 ± 3 versus 17 ± 2 ; 2 weeks 37 ± 3 versus 31 ± 4).

DISCUSSION

Carbonyl groups in proteins

Carbonyl groups are only one by-product resulting from oxidative attack on proteins [18], but, since it is not

Fig. 2. Comparison between the ratio of protein thiol pools and the ratio of glutathione pools

The data are expressed as the mean ratio of pmol of protein thiol per nmol of protein-SSG to pmol of GSH per nmol of GSSG (measured as GSH equivalents as described in the Materials and methods section), $\pm$ S.E.M. as calculated for individual samples ($n = 3-4$): * significantly different from normal birds ($P < 0.05$). ▨, Normal; ■, dystrophic.

known with what frequency the production of a single carbonyl residue on a typical protein alters its function, may signal the occurrence of significant damage. There was a greater content of protein carbonyl groups in dystrophic pectoralis major muscle. Both normal and dystrophic muscle contained only about 1 carbonyl residue per 10000 amino acids. This value is similar to that reported for various proteins from other species [12], but is far less than one carbonyl group in an average-sized protein, and would appear unlikely to produce significant physiological consequences. The greater protein carbonyl content in dystrophic pectoralis major muscle appeared to result from decreases in the carbonyl groups during development in normal, but not dystrophic, animals, and did not appear to be a secondary artifact of other physiological changes.

GSH and GSSG

Increased GSH has been indirectly shown to occur in humans with Duchenne muscular dystrophy [19]. More direct assays have demonstrated increases in GSH in dystrophic chicken muscle [20], but interpretation of the results is complicated by secondary pathological changes in the 4–12-month-old chickens used [9]. Using improved methodology, we observed significantly higher contents of GSH in dystrophic pectoralis major muscle at 1, 2 and 4 weeks, and of GSSG at 4 weeks of age, which were superimposed on a general trend towards decreases in GSH during this period of development. Concluding that the increased GSSG in dystrophic pectoralis major muscle is due to increased oxidative stress may be premature, however, since the GSSG content relative to GSH was unchanged.

The contents of GSSG reported here are lower than in many previous reports concerning muscular dystrophy. Artifactual oxidation of GSH during tissue preparation is a severe problem [13], and it is not possible to assure that the contents of GSSG reported represent the actual value *in vivo*. The GSSG values reported here represent 1–4 % of the total glutathione pools, which matches estimates currently accepted for most normal tissues. On the basis of thermodynamic calculations involving the levels of substrates and enzymes utilized in its reduction, GSSG should represent only around 0.01 % of the glutathione pool [20a]. Nevertheless, since factors such as sub-maximal enzyme activity and compartmentalization are not considered in such calculations, the reported values of GSSG could reflect its actual content. More importantly, comparisons of the GSSG content in tissues prepared by identical techniques may still yield useful information.

Protein thiol groups and mixed disulphides

The content of thiol groups in acid-precipitable proteins is lower than normal in muscle of both dystrophic chickens and mice (Table 2; [21–23]). In chickens, this decrease correlates with a decreased specific activity of the thiol-containing enzymes creatine kinase and glyceraldehyde-3-phosphate dehydrogenase in pectoralis major, but not in other muscles [22]. Tissue protein thiols and disulphides are thought to be in equilibrium with the GSH–GSSG redox pair [3]. However, a decrease in hepatic protein thiols is not clearly linked to GSH depletion [24].

A less developed state of the muscle, or a shift in its biochemical profile to one of a slow-twitch fibre, have

been suggested as alternative explanations for the changes seen in dystrophic muscle. The significantly lower contents of free thiol groups in the muscle proteins of dystrophic birds could be caused by oxidation of the thiol residues. However, since the thiol content of muscle proteins increases with age and is normally lower in soleus muscle than in similarly aged pectoralis major muscle, the lower contents in dystrophic pectoralis major may reflect a partial change of its properties to those of the slow-twitch muscle type, or may simply be due to slower development.

The content of protein–GSH mixed disulphides in the dystrophic pectoralis major muscle from 4-week-old birds, but not in other tissues, was greatly increased. Although this increase may result from an oxidative stress, it is again possible that it reflects a partial change in metabolism of the dystrophic pectoralis major muscle to resemble that of slow-twitch fibres, which normally have significantly higher contents of these mixed disulphides. A maximum of 1 protein–GSH mixed-disulphide group existed for every 4000 free thiol groups in pectoralis major muscle. Thus the increased mixed-disulphide content could not account for the apparent loss of protein thiol groups in dystrophic muscle, and may have no significant physiological consequences [3]. But, like the carbonyl groups, protein–GSH mixed disulphides would only represent a fraction of the possible end products of protein thiol-group oxidation [13], and may occur in much higher amounts in a small pool of proteins, where their effects could be substantial.

In comparison with a previous report on the thiol pools of dystrophic muscle [23], the absolute amounts of mixed disulphides reported here are very low. This discrepancy is likely to be due to the differences in techniques used to measure the mixed disulphides, including analysis of mixed disulphides in addition to GSH, homogenization with reagents that could not completely prevent the oxidation of thiol groups, and the use of reducing agents (such as dithiothreitol and borohydride) which could produce spuriously high estimates of released thiol groups. Since almost all possible artifacts of such analysis tend to cause over-estimations of the mixed-disulphide content, the low values reported here are more likely to be indicative of true levels.

Relationship between glutathione and protein thiol pools

In every normal and dystrophic tissue examined, except pectoralis major muscle from normal 4-week-old birds, the ratio of free thiols to protein–GSH mixed disulphides was 20–80 times the ratio of GSH to GSSG. Normal pectoralis major muscle had a free-thiol/protein–GSH mixed-disulphide ratio that was over 200 times the ratio of GSH to GSSG. This finding was unexpected, since both ratios would be expected to reflect the occurrence of thiol oxidation in a tissue, and the pools are thought to be in equilibrium. However, the close parallel between the contents of GSSG and protein–GSH mixed disulphides may indicate that the absolute, rather than relative, content of GSSG in a tissue is a determining factor in the oxidation state of at least some protein thiol groups.

Several mechanisms could be involved in the unique thiol-oxidation status of normal pectoralis major muscle. This tissue may form protein–GSH mixed disulphides at a much lower rate, perhaps because of its scarcity of mitochondria or other sources of endogenous oxidative

stress. Higher rates of mixed-disulphide formation in dystrophic pectoralis major muscle, with only limited or indirect effects on the glutathione thiol pool, could also produce the observed difference. In this case, the fact that the dystrophic muscle shows a relationship between the pools that resembles that in all other tissues would be coincidental. Alternatively, a process or pathway in normal pectoralis major muscle which preferentially maintains the free thiol groups of proteins, or regenerates them from oxidized forms, may be absent from or less active in dystrophic birds and other tissues. Interestingly, the ratio between the thiol pools in normal pectoralis major muscle from younger birds was more like that of other tissues, suggesting there is a developmentally regulated appearance of such a characteristic.

The changes in tissue thiols and protein carbonyl groups reported here provide additional indications that oxidative processes are occurring in dystrophic muscle. The magnitude of these changes was relatively small, and pathological changes were not evident in the young tissue studied. However, over succeeding weeks and months it is possible that the accumulation of oxidative injury could explain some of the damage characteristic of dystrophic muscle.

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