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Free Radical-Mediated Effects on Skeletal Muscle Protein in Rats Treated with Fe-Nitrilotriacetate

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Changes in protein conformation and proteolysis in skeletal muscle of rats were studied by the induction of oxidative stress induced *in vivo* by ferric nitrilotriacetate (FeNTA) treatment. Useful indices of protein modification, including both protein carbonyl content and fluorescence intensity of protein hydrolysate in skeletal muscle, were increased 3 h following FeNTA treatment to rats. Western blot using anti-dinitrophenyl antibody showed oxidative modification of actin and myosin in myofibril by FeNTA. These results demonstrated that muscle proteins were modified after radical attack induced by an iron overload. Furthermore, oxidative stress induced by iron overloading resulted in enhanced degradation of myofibrillar proteins. It is suggested that muscle proteins which have been modified by oxidative stress undergo rapid removal.

Free radicals produced by either natural consequence of cellular metabolism or as a result of pathological events are known to attack lipids, pigments, nucleotides and proteins and result in cellular injury. Oxidative damage of proteins can result in reduced biological function and enhanced susceptibility to proteolysis (1-3). Proteins modified by free radical attack are easily degraded by proteasomes (3,4) and some proteins with characteristically long half lives, such as crystalline in lens, are susceptible targets for free radical attack (4,5). Skeletal muscle comprises about 40% of body weight and has very important roles in organ and whole body protein and amino acid metabolism. The turnover rate of skeletal muscle proteins is comparatively slower than other tissue proteins (6) and therefore potentially more susceptible to protein fragmentation or conformational changes induced by free radicals. There is a paucity of information available concerning the specific changes occurring in modified skeletal muscle proteins following *in vivo* exposure to free radical-generating systems.

Free iron is an important catalyst in the generation of reactive oxygen species. Iron overload in animals leads to oxidative stress against cells and tissues (7). In this study, we utilized a synthetic tricarboxylic acid, nitrilotriacetic acid which forms a water soluble chelate complex with iron producing ferric nitrilotriacetate (FeNTA), a known renal carcinogen and hepatic tumor promoter, which can modify both protein and DNA (8-10). The purpose of this study was to demonstrate the effect of FeNTA-induced oxidative stress on skeletal muscle protein modifications and rate of muscle protein degradation *in vivo*.

MATERIALS AND METHODS

Anti-dinitrophenyl antibody (IgE) and bovine serum albumin (Fraction V) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Rat anti-mouse IgE was obtained from Meiji Milk Products Co. (Tokyo, Japan). Biotinylated anti-mouse IgE was prepared by coupling anti-mouse IgE (10 mg) with *N*-hydroysuccimidobiotin (10 mg, E-Y Laboratories, SanMateo, CA, USA) at pH 9.5 overnight at 4°C. Biotin-avidin peroxidase complex was obtained from Vector Laboratories, Inc., CA, USA). Chemiluminescence was performed using an ECL kit (Amersham, UK). All other chemicals were purchased from Wako Pure Chemical Co. (Osaka, Japan).

In experiment 1, twenty male, 5 week old Sprague-Dawely rats were obtained from Clea Japan Inc. (Tokyo, Japan) and caged individually in a room with a 12/12 h light-dark cycle. Animals were fed commercial lab chow (F-2, Funabashi Farm, Chiba, Japan) and distilled water *ad libitum* through the course of the experiment. Ferric nitrilotriacetate (FeNTA) was prepared by the method of Goddard *et al.* (11) and administered to rats by an intraperitoneal injection (Fe 100 μmole/kg body weight). Rats were sacrificed at 1.5, 3

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Abbreviations used: CBB, Coomassie brilliant blue; DNP, 2,4-dinitrophenyl; DNPH, 2,4-dinitrophenylhydrazine; EDL, extensor digitorum longus; FeNTA, ferric nitrilotriacetate; MeHis, 3-methylhistidine; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TBARS, 2-thiobarbituric acid-reactive substance.

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and 6 h after FeNTA injection. Control rats were injected with saline and sacrificed immediately thereafter. The gastrocnemius, soleus and right extensor digitorum longus (EDL) muscles were removed from rat hind limb immediately following sacrifice and kept in ice cold buffer until used. Left EDL muscle were used to measure the rate of protein degradation. In experiment 2, twelve male, 5 week old Sprague-Dawely rats were maintained as outlined above. Six animals were given saline and an intraperitoneal injection of FeNTA (100 μmol/kg body weight), respectively, prior to being sacrificed 3 hr after the injection. Gastrocnemius soleus and EDL muscles were removed from hind limb. Left soleus and EDL muscles were used to measure the rate of protein degradation.

The rate of protein degradation was measured by a procedure described previously (12). Briefly, soleus and EDL muscles were removed from the hind limb and incubated with Krebs-Ringer bicarbonate buffer (pH 7.5) containing 10 mM glucose and 0.5 mM cycloheximide (incubation medium) for 30 min at 37°C. Following incubation, isolated muscles were incubated again for 2 h at 37°C. The release of tyrosine and 3-methylhistidine (MeHis) from isolated muscle into the incubation medium were measured by fluorometric (13) and HPLC (14) methods, respectively. Since tyrosine is not metabolized in the muscle cell, its release from muscle under the presence of protein synthesis inhibitor, cycloheximide indicates muscle protein degradation. MeHis is known as an index of myofibrillar protein degradation, therefore its release can be used as an index of myofibrillar protein degradation (12). We measured tyrosine release from EDL muscle in Experiment 1 and tyrosine and MeHis release from both soleus and EDL muscles in Experiment 2.

Muscle tissues were homogenized in 0.1M sodium phosphate buffer, pH 7.5. 2-Thiobarbituric acid-reactive substance (TBARS) of muscle was determined according to the method of Mihara and Uchiyama (15). Protein carbonyl content of both the homogenate was measured using 2,4-dinitrophenylhydrazine (DNPH) (16). The carbonyl content was calculated as molar absorbance, 21,000 $M^{-1} \cdot cm^{-1}$. Protein was measured by the method of Markwell *et al.* (17) using bovine serum albumin as the standard. Fluorescence intensity of muscle protein hydrolysate was measured on trichloroacetic acid insoluble fractions of muscle homogenate treated with 3.3 N *p*-toluenesulofonic acid at 110°C for 24 h using a model FP-4 spectrofluorometer (Japan Spectro Scopic Co., Tokyo, Japan) with an excitation wave length of 325 nm and an emission wave length of 410 nm (18).

Western blots were performed on tissue homogenate as described by Shacter *et al.* (19) with slight modifications. The fractionation of muscle homogenate was performed by centrifugation at 700 *g* for 15 min. The supernatant was then centrifuged at 10,000 *g* for 15 min. 2,4-Dinitrophenylhydrazine (2 mM DNPH in 2 M HCl) was added to the trichloroacetic acid insoluble protein of each fraction and reacted for 1 h at room temperature. The sample was washed by ethanol/ethylacetate (1/1) three times and finally dissolved in sample buffer for preparation for sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Duplicate SDS-PAGE was performed in a 7.5-15% linear gradient gel (Biokraft, Tokyo, Japan). One gel was stained with Coomassie Brilliant Blue (CBB) and proteins identified on the other gel were transferred to a nitrocellulose membrane. Blots were blocked with skim milk and incubated sequentially with anti-DNP antibody, biotinylated rat anti-mouse IgE and a biotin-avidin-peroxidase complex. DNP-protein containing bands were visualized by chemiluminescence and exposure to x-ray film.

Results are presented as means and standard error. The effects of time were tested by one-way analysis of variance and Dunnet multiple comparison test (GraphPad InStat Software version 2.03, 1995, San Diego, CA). In the second experiment significance was tested by Student's *t*-test. Significance was considered present at $p<0.05$.

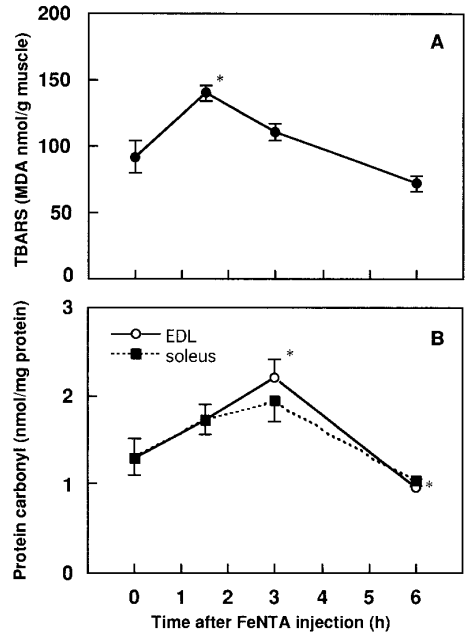

FIG. 1. TBARS of gastrocnemius muscle (A) and protein carbonyl contents of extensor digitorum longus (EDL) and soleus muscle (B) from rats injected with FeNTA. Values represent mean ± SEM (n=5).

RESULTS

In experiment 1, the TBARS concentration in gastrocnemius muscle reached a maximum value 1.5 hr after FeNTA injection and then subsequently returned to control levels (Fig. 1A). Protein carbonyl content of both soleus and EDL muscle were elevated up to 3 h after FeNTA injection before returning to control levels (Fig. 1B). These results indicate that muscle proteins were modified by free radicals generated from Fe overload induced by FeNTA injection. EDL muscle showed a 70% increase in protein carbonyl content at 3 h following FeNTA injection, compared to 50% increase in soleus muscle.

In experiment 2, fluorescence intensity was the same for both EDL and soleus muscle samples collected from control animals. Rats treated with FeNTA exhibited significantly ($P<0.05$) greater fluorescence intensity in EDL muscle (70%) only (Fig. 2). The SDS-PAGE pattern of DNP-muscle proteins stained by CBB and anti-DNPH antibody is shown in Figures 3A and B. There were no differences in protein patterns resulting from CBB staining in fractionated muscle proteins collected from FeNTA treated animals. In contrast, staining the same samples with anti-DNP antibody enabled detection of a FeNTA induced oxidatively modified proteins with a molecular weight of 42 and 200 kDa in the 700 *g* pellet which corresponded to actin and myosin. These results demonstrated that myofibrillar proteins can be

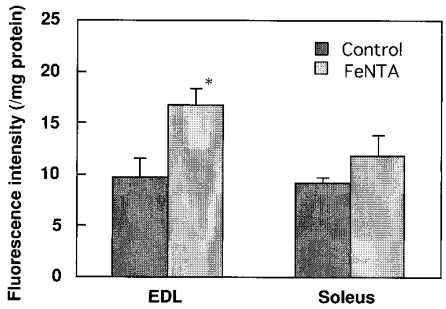

FIG. 2. Fluorescence intensity of skeletal muscle protein hydrolysate from rats 3 hours after FeNTA treatment. Values represent mean ± SEM (n=5). * denotes $p<0.05$ versus control.

modified *in vivo* by FeNTA treatment. Treatment of the supernatant fraction with anti-DNP antibody also produced detectable protein bands with molecular weights (42kD) that corresponded to salcoplasmic proteins. All fractions contained a strongly stained protein band at 66 kDa, which was not identified.

In the Experiment 1, we also measured the rate of tyrosine release from isolated EDL muscle. The maximum rate of tyrosine release was reached at 3 h after FeNTA injection (Fig. 4A), suggesting an increase in the rate of total muscle protein degradation by FeNTA injection. Measuring the degradation rate of soleus and EDL muscle protein by the presence of tyrosine and MeHis release into incubation medium (Experiment 2) revealed unique differences between different muscle sources (Figures 4B and C). Significant ($P<0.05$) increases in both measures of protein degradation were observed in soleus and EDL muscle from FeNTA treated rats, compared to control animals.

DISCUSSION

Several studies examining the modification of proteins by oxidative stress have identified changes in

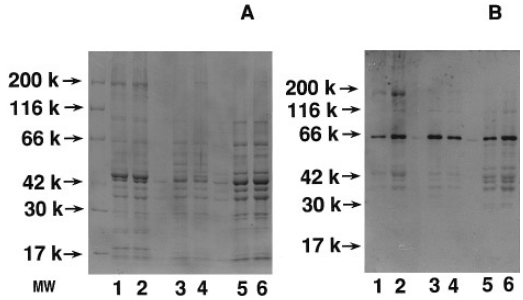

FIG. 3. Western blot analysis of skeletal muscle protein fractions collected after 700 *g* and 10,000 *g* centrifugation. A, CBB stain; B, immunoblot with anti-DNP. Lanes 1 and 2, 700 *g* pellet; lanes 3 and 4, 10,000 *g* pellet; lanes 5 and 6, 10,000 *g* supernatant. Lanes 1,3, and 5, control; lanes 2,4, and 6, FeNTA treatment.

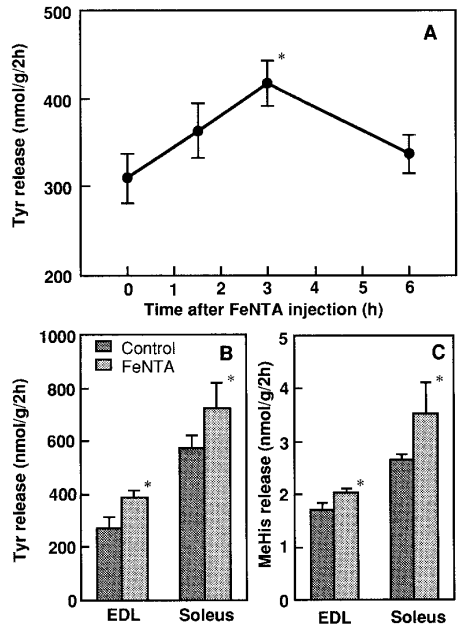

FIG. 4. Tyrosine and 3-methylhistidine (MeHis) release from isolated rat EDL and soleus muscle. A, tyrosine release from EDL muscle after FeNTA injection. B, tyrosine release from EDL and soleus muscle 3 h after FeNTA injection. C, MeHis release from EDL and soleus muscle 3 h after FeNTA injection. Values represent mean ± SEM (n=5 or 6). * denotes $p<0.05$ versus 0 h (A) or control (B and C).

amino acids that resulted in fragmentation and conformational modifications of proteins and subsequent changes in protein functionality (18,19,21). The monitoring of florescence intensity in skeletal muscle protein samples in the present study (Fig. 2) was one indicator used to estimate oxidative damage of protein by the modification of amino acids phenylalanine and tyrosine and subsequent formation of fluorescent products *o*-tyrosine and dityrosine (6,18). The increase in fluorescence intensity of skeletal muscle protein from rats exposed to FeNTA in this study, coupled with a similar enhancement in protein carbonyl content in the same tissue (Fig. 1B), provided strong evidence for damaged skeletal protein by *in vivo* FeNTA treatment. Other workers have demonstrated an induced accumulation of protein carbonyl content in cytosolic fractions collected from rat kidney (8), which is consistent with our data that showed maximal accumulation of protein carbonyl content after only 3 h following exposure of rats to FeNTA (Fig. 1B). On the other hand, the fact that TBARS values reached highest levels after only 1.5 h after FeNTA exposure (Fig. 1A), suggests that peroxy-radicals in addition to oxygen free radicals may have also been involved in FeNTA induced protein oxidation. Lipid radicals have been reported to damage proteins (22) and in particular can result in protein fragmentation (23).

An important finding of this study was the increased

protein carbonyl content and fluorescence intensity was more pronounced in EDL (fast twitch, glycolytic muscle) compared to soleus (slow twitch, oxidative muscle) in FeNTA treated rats. The fact that we could measure oxidative stress in both muscle types in our study reflects the relatively high oxygen consumption and metabolic activity (e.g. contractility) that would be expected in active skeletal muscle (24). Furthermore, the different response to oxidative stress in the two muscle types observed herein, reflects a potential for a relatively greater resistance against protein free radical damage for soleus muscle. This suggestion is supported by the findings that soleus muscle has higher glutathione peroxidase activity (25,26) and thus greater affinity to potentially detoxify free radicals (27).

Although, other workers have shown an increase in protein carbonyl content in dystrophic chickens caused by oxidative stress (28), it is difficult to evaluate which protein(s) are modified when using only a colourmetric determination of protein carbonyl content. The use of a specific antibody against 2,4-dinitrophenyl group enabled further characterization of the oxidation of muscle protein oxidation in this study (Fig. 3). Myosin and actin from rats injected with FeNTA gave strong responses to specific antibody, indicating that these two myofibril proteins were profoundly modified by free radicals induced from in vivo oxidative stress. Although this observation supports previous *in vitro* findings for FeNTA or hydrogen peroxide induced protein fragmentation (e.g. bovine serum albumin (23,29)), we were not able to differentiate whether the positive responses of minor bands corresponding to 80 kDa and 40 kDa on the Western blot were produced from fragmentation or proteolysis. The latter is a possibility since oxidatively damaged protein can be degraded by proteinases, especially by the ubiquitin-proteasome system (3,4,20, 30,31). An increase in surface hydrophobicity has been used to demonstrate intracellular protein degradation using the example of hemoglobin breakdown by proteasome during exposure to hydroxyl radicals (30). Other studies have reported an increase in ubiquitin-protein conjugate during recovery of bovine lens epithelial cells following an oxidative stress induced by hydrogen peroxide (31). The protein carbonyl content in EDL (Fig. 1B) and the rate of tyrosine release from EDL muscle (Fig. 4A) reached the maximum values at 3 h after FeNTA injection. Therefore, it is suggested that the protein modification by free radical may relate with its proteolysis, although in the present study it could not be determined whether the free radical mediated change in substrate conformation, or the activation of the ubiquitin-proteasome system was the rate limiting step in subsequent intracellular degradation of oxidatively damaged muscle protein. The possibility that our findings could be attributed to a direct stimulation of proteolysis by iron is an alternative, less likely, explanation of our results since no intracellular proteolytic activities are stimulated by iron.

In conclusion, the combined use of MeHis and tyrosine release enabled the demonstration that myofibrillar muscle proteins are degraded more rapidly when oxidatively modified *in vivo*. The chemical nature of protein carbonyls detected in western blot procedures of whole muscle extracts provided information concerning the mechanism for the rapid free radical mediated cellular protein damage observed herein; however further studies are required to elucidate how these observations are involved with events associated with intracellular proteolysis and protein turnover.

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REFERENCES

1. Stadtman, E. R., and Oliver, C. N. (1991) *J. Biol. Chem.* **266,** 2005–2008.
2. Stadtman, E. R. (1990) *Biochemistry* **29,** 6323–6331.
3. Davies, K. J. A. (1993) *Biochem. Soc. Trans.* **21,** 346–353.
4. Giulivi, C., Pacifici, R. E., and Davies, K. J. A. (1994) *Arch. Biochem. Biophys.* **311,** 329–341.
5. Garner, M. H., and Spector, A. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 1274–1277.
6. Waterlow, J. C., Garlick, P. J., and Millward, D. J. (1978) *in* Protein Turnover in Mammalian Tissues and Whole Body, North-Holland Publishing, Amsterdam.
7. McCord, J. M. (1996) *Nutr. Rev.* **54,** 85–88.
8. Uchida, K., Fukuda, A., Kawakishi, S., Hiai, H., and Toyokuni, S. (1995) *Arch. Biochem. Biophys.* **317,** 405–411.
9. Ito, H., Shioda, T., Matsura, T., Koyama, S., Nakanishi, T., Kajiyama, G., and Kawaskai, T. (1994) *Arch. Biochem. Biophys.* **313,** 120–125.
10. Iqbal, M., Giri, U., and Athar, M. (1995) *Biochem. Biophys. Res. Commun.* **212,** 557–563.
11. Goddard, J. G., Basford, D., and Sweeney, G. D. (1986) *Biochem. Pharmacol.* **35,** 2381–2387.
12. Nagasawa, T., Yoshizaawa, F., and Nishizawa, N. (1996) *Biosc. Biotech. Biochem.* **60,** 501–502.
13. Waalkes, T. P., and Udendfriend, S. (1957) *J. Lab. Clin. Med.* **50,** 733–736.
14. Wassner, S. J., Schlitzer, J. L., and Li, J. B. (1980) *Anal. Biochem.* **104,** 284–289.
15. Mihara, M., and Uchiyama, M. (1978) *Anal. Biochem.* **86,** 271–278.
16. Oliver, C. N., Ahn, B., and Moerman, E. J. (1987) *J. Biol. Chem.* **262,** 5488–5491.
17. Markwell, M. A., Hass, S. M., Bieber, L. L., and Tolbert, N. E. (1978) *Anal. Biochem.* **87,** 206–210.
18. Davies, K. J. A., Delsignore, M. E., and Lin, S. W. (1987) *J. Biol. Chem.* **262,** 9902–9907.
19. Shacter, E., Williams, J. A., Michael, L., and Levine, R. L. (1994) *Free Rad. Biol. Med.* **17,** 429–437.

20. Stadtman, E. R. (1990) *Free Rad. Biol. Med.* **9,** 315–325.
21. Davies, K. J. A. (1987) *J. Biol. Chem.* **262,** 9895–9901.
22. Tappel, A. L. (1975) *in* Pathobiology of Cell Membranes, pp. 145–170, Academic Press.
23. Hunt, J. V., Simpson, J. A., and Dean, R. T. (1988) *Biochem. J.* **250,** 87–93.
24. Witt, E. H., Reznick, A. Z., Viguie, C. A., Starke-Reed, P., and Packer, L. (1992) *J. Nutr.* **122,** 766–773.
25. Asayama, K., Dettbarn, W. D., Burr, I. M. (1986) *J. Neurochem.* **46,** 604–609.
26. Ji, L. L., Fu, R., and Mitchell, E. W. (1992) *J. Appl. Physiol.* **73,** 1854–1859.
27. Yuan, Y. V., and Kitts, D. D. (1996) *in* Natural Antioxidants, pp. 258–270, AOCS Press, Champaign, IL.
28. Murphy, M. E., and Kehrer, P. (1989) *Biochem. J.* **260,** 359–364.
29. Ogino, T., and Okada, S. (1995) *Biochim Biophys. Acta* **1245,** 359–365.
30. Pacifici, R. E., Kono, Y., and Davies, K. J. A. (1993) *J. Biol. Chem.* **268,** 15405–15411.
31. Shang, F., and Taylor, A. (1995) *Biochem. J.* **307,** 297–303.