Induction of Oxidatively Modified Proteins in Skeletal Muscle by Electrical Stimulation and Its Suppression by Dietary Supplementation of (−)-Epigallocatechin Gallate

Takashi Nagasawa,† Hiromichi Hayashi, Naoko Fujimaki, Naoyuki Nishizawa, and David D. Kitts*

Department of Bioscience and Technology, Faculty of Agriculture, Iwate University, Morioka, Iwate 020-8550, Japan
*Food, Nutrition and Health, Faculty of Agricultural Sciences, The University of British Columbia, Vancouver, B.C. V6T 1W5, Canada

Received November 19, 1999; Accepted December 28, 1999

The oxidative stress produced by electrical stimulation-induced muscle contraction was examined in the skeletal muscle proteins of rats that had been fed on the dietary flavonoid, (−)-epigallocatechin gallate (EGCg). Electrical stimulation of the rat leg muscle every second day for a two-week period resulted in an increased (p < 0.05) muscle weight and accumulation of oxidatively induced modified proteins. Similar stimulation conducted every day for only one week had no effect on the muscle weight or protein oxidation, although the rate of protein degradation increased. Rats fed on a 20% casein diet supplemented with 0.1% EGCg for 2 weeks responded to the electrical stimulation of muscle contraction by reducing the increased muscle protein carbonyl content when compared to their counterparts fed on a control diet. There was no change in activity of antioxidative enzymes in muscle tissue of the EGCg-fed rats receiving electrical stimulation. The results of this study show that the antioxidative property of EGCg was effective for suppressing oxidative modification of the skeletal muscle protein induced by electrical stimulation. This finding demonstrates that EGCg has a beneficial effect in vivo on the free radical-mediated oxidative damage to muscle proteins.

Key words: muscle; oxidatively modified protein; protein degradation; flavonoid; free radicals

Free radicals or reactive oxygen species (ROS) modify the cellular lipid, protein and nucleic acid components and lead to cellular damage and dysfunction. In vitro studies have shown that the oxidation of proteins includes chemical modification of amino acids and protein fragmentation or aggregation (cross-linking), thus reducing functionality. Modified proteins resulting from oxidation reactions are sensitive to proteinases, particularly to proteasome, which represents one cellular defense system against free radicals. The modification of proteins by ROS has been associated with the development of chronic diseases, such as atherosclerosis, diabetes and neurodegenerative disorders. A characteristic of oxidatively modified protein (OMP), as shown for collagen and crystallin, is the unusually slow turnover rate. This observation could have significance to skeletal muscle protein, which has a relatively slower turnover rate than splanchic tissue proteins, and may explain the accumulation of OMP in skeletal muscle that has been reported in several studies including exercise. Since skeletal muscle proteins are most abundant in the body, the accumulation of OMP in muscles could reduce the muscle function and contribute to atrophy.

The purpose of this study was to determine initially if OMP could be derived from contracted skeletal muscle by following different protocols for electrical stimulation, and whether this form of oxidative stress would result in protein degradation. In addition, we also tested the effectiveness of the plant polyphenol, (−)-epigallocatechin gallate (EGCg), which is derived from green tea and noted for its antioxidative activity, to decrease the oxidation of muscle proteins.

Materials and Methods

Materials. EGCg was kindly provided by Dr. Hara (Mitsui Norin Co. Fujieda, Shizuoka, Japan). Glutathione reductase and NADPH were purchased

† To whom correspondence should be addressed. Tel: +81-19-621-6169; Fax: +81-19-621-6262; E-mail: tnaga@iwate-u.ac.jp

Abbreviations: DPNH, 2,4-dinitrophenylhydrazine; EGCg, (−)-epigallocatechin gallate; GPX, glutathione peroxidase; GST, glutathione-S-transferase; OMP, oxidatively modified protein; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances
from Oriental Yeast Co. (Tokyo, Japan), and xanthine oxidase was purchased from Wako Pure Chemical Industries (Osaka, Japan). tert-Butyl-hydroperoxide was purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals used were of chemically pure grade obtained from commercial sources.

**Effect of the interval of muscle electrical stimulation on protein oxidation and degradation.** Twelve male Sprague-Dawley rats of 5 and 6 weeks of age from Clea Japan (Tokyo, Japan) were used. The animals were housed individually in stainless steel cages and given free access to a commercial laboratory diet and water. Six rats (6 weeks of age) were electrically stimulated in the hindlimb muscle every day for a week (i.e. 7 times/week), this being classified as the high-frequency treatment. The other six rats (5 weeks of age) were given an electrical stimulus every second day for 2 weeks (i.e. 7 times/2 weeks), this being classified as the low-frequency treatment. The procedure for the electrical stimulation of leg muscle was performed according to the method of Inoue et al.\textsuperscript{29} The rats were anesthetized with sodium pentobarbital, and a needle-electrode attached to an electrical stimulator (SEN-2101, Nihon Koden, Tokyo, Japan) was inserted into the left gastrocnemius muscle. Rectangular pulses of 2 ms duration (10 V at 100 Hz) were delivered to induce a tetanic contraction. The stimulation pulses were given for 2 s and followed by a 5 s rest. One set consisted of 10 stimulations. Three sets of electrical stimulation were given at 5-min intervals to each rat every day the high-frequency treatment group. A needle of the same size as the electrode, but delivering no electrical charge, was also inserted into the right gastrocnemius muscle of the animal, thus enabling each rat to be used as its own control. The gastrocnemius and soleus muscles, with and without stimulation, were removed 3 h after the final electrical stimulation. The soleus muscles were used for measuring the protein degradation, while the gastrocnemius muscles were immediately frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\) until needed for analysis. The animal care protocol for these experiments was approved by the Iwate University Animal Research Committee under the Guidelines to Animal Experiments in Faculty of Agriculture at Iwate University.

**Effect of the dietary antioxidant on the protein oxidation and degradation reactions.** Twelve male, 5-week-old Sprague-Dawley rats were purchased from Clea Japan and cared for under the conditions already described. The animals were allowed free access to water and the commercial feed for 3 d and then randomly divided into 2 groups. The control group was fed on a 20% casein diet according to the AIN 76 composition, while the EGCG-supplemented group was fed on a 20% casein diet containing 0.1% EGCG. All animals were allowed free access to water and were fed experimental diets for 2 weeks. The hindlimb muscle in the left leg of each rat was electrically stimulated every second day for 2 weeks, while non-electrical stimulation was applied to the right leg muscle of each rat during the feeding experiment. The procedures for electrical and non-electrical stimulation of the individual legs were the same as those already described. Three hours after the last stimulation, the rats were sacrificed, and the gastrocnemius and soleus muscles were removed from both hindlimbs. The soleus muscle was used for measuring protein degradation, whereas the gastrocnemius muscle was immediately frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\) until needed for analysis.

**Thiobarbituric acid reactive substances and protein carbonyl content.** Lipid peroxidation was evaluated by the presence of thiobarbituric acid reactive substances (TBARS) which were measured according to the method of Uchiyama and Mišara,\textsuperscript{23} using tetraethoxypropane as the standard. The level of TBARS is expressed as the malondialdehyde equivalent. The protein carbonyl group content was used as an index of the oxidative modification to protein according to the method of Levine et al.,\textsuperscript{23} using 2,4-dinitrophenylhydrazine (DNPH). The protein carbonyl content was calculated from the 370 nm absorbance by using a molar absorbance of 21,000 \(\text{M}^{-1}\) \(\text{cm}^{-1}\). The protein content was measured by a modification of the Lowry method described by Marcwell et al.\textsuperscript{25} Bovine serum albumin was used as the standard.

**Rate of muscle protein degradation.** Soleus muscles with and without electrical stimulation were used to measure the rate of protein degradation.\textsuperscript{24} The muscles were immediately transferred to a Krebs-Ringer bicarbonate buffer containing 10 mm glucose and 0.5 mm cycloheximide and bubbled with 95% O\textsubscript{2}-5% CO\textsubscript{2} and were incubated at 37°C for 30 min before being transferred to a fresh buffer for further incubation for 2 h. After the incubation, the tyrosine concentration was measured in the incubation buffer by a fluorometric method.\textsuperscript{25}

**Antioxidative enzyme assay.** Gastrocnemius muscle (100 mg) was homogenized with 5 volumes of 10 mm Tris-HCl, 0.5 mm EDTA buffer (pH 7.5), before being centrifuged at 750 \(\times g\) for 20 min. The resulting supernatant was used for measuring the superoxide dismutase (SOD), glutathione peroxidase (GPX) and glutathione-S-transferase (GST) activities. SOD activity was measured by the xanthine-xanthine oxidase-nitrobluetetrazolium reaction,\textsuperscript{26} one unit of SOD activity being defined as the amount of enzyme that inhibited 50% the rate of reduction of nitro-
luteotrazolium. The activity of GPX with tert-butyl-hydroperoxide as a substrate was measured spectrophotometrically by monitoring glutathione disulfide formation through the concomitant oxidation of NADPH.\textsuperscript{20} The activity of GST was measured according to the method of Habig et al.,\textsuperscript{20} using 1-chloro-2,4-dinitrobenzene as a substrate.

**Statistical analysis.** Raw data from each animal, which represented its own control, was transformed to a mean and SE, and a two-tailed paired \( t \)-test was applied to the difference between the stimulated and non-stimulated means by using GraphPad InStat software (v. 2.03, 1995, San Diego, CA, U.S.A.). A two-tailed unpaired \( t \)-test was also used for determining the effect of dietary ECGg supplementation. Statistical significance was set at \( p < 0.05 \).

**Results**

**Effect of the frequency of applying an electrical stimulation on protein oxidation and degradation**

The final body weight was not significantly different between the animals receiving high- and low-frequency treatment (262 ± 7 vs 271 ± 7), which indicates that the experimental manipulation had no effect on animal growth, and both groups seemed to eat a similar amount of laboratory feed. The gastrocnemius and soleus muscles of the rats receiving the low-frequency treatment were heavier significantly (\( p < 0.05 \) by the paired \( t \)-test) than the control muscles from the same rat that had not been stimulated. In contrast, the high-frequency treatment had no significant effect on muscle weight (Table 1). The changes in both muscle weights with the low-frequency treatment corresponded to a significantly (\( p < 0.05 \)) higher protein carbonyl content than that in control muscle that had not received electrical stimulation (Table 1), whereas the high-frequency treatment did not affect the protein carbonyl content.

In the present study, protein degradation in the soleus muscle only was measured by the amount of tyrosine recovered in a physiological buffer containing cycloheximide to inhibit protein synthesis. Thus, the concentration of tyrosine in the buffer reflected the rate of tyrosine release and whole-muscle protein degradation, since tyrosine is not metabolized in the muscle cells unless being used for protein synthesis.\textsuperscript{20} Of particular interest was the observation that the animals receiving the high-frequency treatment exhibited a higher rate (\( p < 0.05 \)) of tyrosine release by the stimulated muscle than by the unstimulated muscle, as was evident from the concentration of tyrosine in the buffer (Table 1). No significant difference in the rate of tyrosine release between the stimulated and unstimulated soleus muscles was observed in the animals receiving the low-frequency treatment, which corresponded to a higher level of OMP in the soleus muscle (Table 1).

**Effect of dietary antioxidant on protein oxidation and degradation**

In this experiment, supplementing with EGCg produced no significant difference in the food intake or body weight gain (Table 2). Although the electrical stimulation applied every second day over a longer duration had no effect on the food intake or body weight gain, it did result in muscle hypertrophy in both the gastrocnemius and soleus muscles of the rats fed on either the control or EGCg-supplemented diet (Table 2). This result indicates that the dietary EGCg intake had no effect on the muscle hypertrophy induced by electrical stimulation with the low-frequency treatment.

<table>
<thead>
<tr>
<th>Table 1. Effect of the Frequency of Electrical Stimulation of Rat Hindlimb Muscles on Muscle Hypertrophy and Oxidative Modification of Proteins</th>
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<tbody>
<tr>
<td>Treatment</td>
</tr>
<tr>
<td>High-frequency treatment</td>
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<td></td>
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<tr>
<td>Muscle weight</td>
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<tr>
<td>Gastrocnemius muscle (g)</td>
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<tr>
<td>(100)</td>
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<tr>
<td>Soleus muscle (mg)</td>
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<td>(100)</td>
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<tr>
<td>Protein carbonyl content (nmol/mg of protein)</td>
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<tr>
<td>Gastrocnemius muscle</td>
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<td>(100)</td>
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<td>Soleus muscle</td>
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<td>(100)</td>
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<tr>
<td>Protein degradation rate (nmol Tyr/g/2h)</td>
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<td>(100)</td>
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\( \text{1 Each value is mean ± SE for 6 rats.} \)

\( \text{2 Each value is relative to the mean value for unstimulated muscle calculated for the same rats.} \)

\* \( p < 0.05 \) vs unstimulated muscle by a paired \( t \)-test.
Reduced Oxidative Damage to Muscle Proteins by EGCG

Table 2. Food Intake, Body Weight and Muscle Weight of Rats Fed on the Control Diet or EGCG-supplemented Diet

<table>
<thead>
<tr>
<th>Diet</th>
<th>Food intake (g/day)</th>
<th>Body weight (g)</th>
<th>Gastrocnemius</th>
<th>Soleus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Unstimulated (g)</td>
<td>Stimulated (g)</td>
</tr>
<tr>
<td>Control</td>
<td>21.3 ± 2.5</td>
<td>240 ± 5</td>
<td>1.41 ± 0.031</td>
<td>1.48 ± 0.04*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(100)</td>
<td>(106 ± 1)2</td>
</tr>
<tr>
<td>Supplemented</td>
<td>22.0 ± 1.9</td>
<td>237 ± 2</td>
<td>1.37 ± 0.02</td>
<td>1.43 ± 0.01*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(100)</td>
<td>(104 ± 2)</td>
</tr>
</tbody>
</table>

1 Each value is mean ± SE for 6 rats.
2 Each value is relative to the mean value for unstimulated muscle calculated for the same rats.
* p<0.05 vs unstimulated muscle by a paired t-test.

Neither the presence of dietary EGCG, nor the application of electrical stimulation influenced the muscle TBARS content above the respective control level (Fig. 1A). The increased protein carbonyl contents of the electrically stimulated gastrocnemius and soleus muscle in the rats fed on the control diet were significantly reduced (p<0.05) in the rats fed on the EGCG-supplemented diet (Fig. 1B), indicating that feeding with EGCG was effective against OMP induced in muscles contracted by the electrical stimulation. Dietary EGCG did not affect the protein degradation in stimulated and unstimulated muscles, although the EGCG supplement decreased the muscle protein degradation (Fig. 2).

The antioxidative enzyme activity measured in skeletal muscle after the electrical stimulation is shown in Fig. 3. No significant difference was observed in the antioxidative enzyme activity between the rats fed on the EGCG-supplemented and control diets, suggesting that dietary EGCG did not affect the antioxidative enzyme activities. Moreover, electrical stimulation produced no effect on the muscle antioxidative enzyme activities.

Discussion

The results of the present study clearly show that electrical stimulation of the skeletal muscle to produce chronic muscle contraction in the rats receiving the low-frequency treatment resulted in a weight gain of the muscle that could be attributed to the accumulation of OMP. Furthermore, the dietary antioxidant, EGCG, was shown to be effective at protecting against OMP in the electrically stimulated muscle.

In our study, the weight gain of the gastrocnemius muscle following the low-frequency treatment increased 4-5%. This finding agrees with former results reported by Inoue et al.,20 who observed a 5.9% increase in gastrocnemius muscle weight after 2 weeks of electrical stimulation which was attributed to hypertrophy of the muscle cells. We extend these findings by reporting that the weight of the soleus muscle also increased after chronic electrical stimulation had been applied to the gastrocnemius muscle (Tables 1 and 2). The soleus muscle, which is located directly interior to the gastrocnemius muscle, also showed hypertrophy. The fact that varying the protocol for applying electrical stimulation to the muscle resulted in a different response in muscle weight in this study is also noteworthy. If we assume that the stimulation increased the rate of muscle protein synthesis, the observed unchanged weight and protein carbonyl content between the stimulated and un-
stimulated muscle in those animals treated every day with an electrical charge may represent a more rapid rate of muscle protein degradation due to the high-frequency treatment. However, the mechanism for the increased rate of protein degradation is unclear. Alternatively, the unchanged rate of protein degradation rate may have caused an increase in those muscle weight in rat with the low-frequency treatment, although OMP was accumulated.

Muscle contraction requires ATP which is produced from the mitochondrial electron transport chain. It is known that small, but significant amounts of electrons which leak from the electron transport chain generate such ROS as hydroxyl radical[29] and the superoxide radical[10,31] which, in turn, contribute to elevating the damage to many tissue components. The ischemia-reperfusion brought on during high-intensity muscle contraction is another example of increased free radical formation. Davies et al. have shown that exercise also represents an important source of free radicals which are involved in the induction of damaged muscle tissue. Exercise is an important form of oxidative stress, due to the increased consumption of molecular oxygen required for respiration and the generation of free radicals. Studies conducted on both rat and human physical exercise have reported with muscle cells increased protein carbonyl content. Moreover, Jackson has also shown that free radical signals detected by electron spin resonance (ESR) were present in the rat gastrocnemius muscle after electrical stimulation. Taken together, these findings support our results that both the gastrocnemius and soleus muscles treated with electrical stimulation produced ROS and accumulated damaged proteins. The lack of any change in the antioxidative enzyme activities (e.g., SOD, GPX, and GST) indicates that resistance to oxidative stress induced by electrical stimulation was not a factor in this study. Several investigators have shown, however that the activities of different antioxidative enzymes were influenced by physical exercise.

In our previous study, we reported an increase in skeletal muscle TBARS prior to a similar elevation in the protein carbonyl content of rats which had undergone oxidative damage due to iron overloading. In the present study, electrical stimulation of the muscle did not result in any increase in TBARS, thereby indicating that the free radicals produced by intense muscle contraction acted directly on the muscle protein, rather than indirectly through the generation of lipid oxidation products such as aldehydes mediated by lipid hydroperoxide. Lipid peroxidation in the muscle has been shown in rats exposed to exhaustive physical exercise with treadmill running. The induction of lipid peroxidation will also produce a loss of protein thiols, a vital component for the secondary protein structure and essential for the molecular arrangement required for Ca²⁺ transport across the
cellular membrane that is involved in the muscle functions. In contrast, high-altitude training\(^\text{14,16}\) and long-term swimming training\(^\text{1}^\text{6}\) by rats have been shown to have no effect on muscle TBARS, although the protein carbonyl content was elevated. It is evident from these studies, as well as ours here, that both the method and conditions used to evoke muscle protein modification due to oxidative stress yield different biochemical characteristics.

Dietary supplementation with an antioxidant such as vitamin E has been reported to attenuate the oxidative damage to muscles that has been induced by exercise.\(^\text{14,16}\) Tea catechins, including EGCG and (−)-epigallocatechin, have been shown to possess antioxidative activity \textit{in vitro}\(^\text{19,40}\) and \textit{in vivo}.\(^\text{41}\) EGCG comprises up to about 50% of the catechin content in tea and has the greatest antioxidative effect of tea catechins.\(^\text{40}\) Our findings complement the many potential health benefits of tea catechins reported by others\(^\text{42,43}\) by showing the effectiveness of EGCG when used as a dietary supplement to decrease OMP in both the gastrocnemius and soleus muscles after muscle contraction induced by electrical stimulation. EGCG is absorbed from the intestine and reacts with free radicals in various tissues;\(^\text{44}\) therefore the presence of this plant polyphenol in muscle tissue directly or within the circulation perfusing the stimulated muscles appears to be involved the mechanism of action for the protective effect against oxidative modification of muscle induced by oxidative stress. Further studies are required to determine if EGCG or other plant polyphenols are indeed retained in muscle tissue.

In conclusion, although moderate physical exercise is recommended to protect against the development of several diseases, including atherosclerosis and hypertension,\(^\text{45}\) excessive muscle contraction resulting in the generation of free radicals can lead to accelerated oxidative modification of muscle protein. Dietary supplementation with an antioxidant such as EGCG was shown to have a beneficial effect in reducing the oxidative stress related to protein oxidation caused by muscle contraction.

Acknowledgments

We thank Drs. T. Fushiki and K. Inoue (Kyoto University) for kind suggestions about electrical stimulation.

References


