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Hydroxyl radical generation in skeletal muscle atrophied by immobilization

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Abstract

Male Wistar rats (15 weeks old), the ankle joints of one hindlimb of which were immobilized in the extended position for 12 days, were injected with salicylate as a trapping agent for hydroxyl radicals before sacrifice. Atrophied and contralateral soleus, typical slow red muscles were collected and their levels of 2,3-dihydroxybenzoic acid (2,3-DHB), one of the main reaction products formed by the attack of hydroxyl radical on salicylate, were determined using high-performance liquid chromatography with an electrochemical detector. There was a significant increase in 2,3-DHB in the atrophied muscle. This result strongly suggests the enhanced generation in vivo of hydroxyl radicals in atrophied muscle.

Key words: Hydroxyl radical; Muscle atrophy; Salicylate

1. Introduction

Oxidative stress causes free radical reactions, such as lipid peroxidation, which have a role in damaging biological structures and cellular functions. It has been more than 10 years since exhaustive exercise was reported to increase oxidative stress in skeletal muscle, this being generally thought to be related to the pathogenesis of exercise myopathy [1]. Previously, we found that muscle atrophy induced by immobilization is also accompanied by oxidative stress; thiobarbituric acid-reactive substance and oxidized glutathione were increased, and total glutathione was decreased in the atrophied muscle [2]. Moreover, in the same report, we proved that such oxidative stress accelerated muscle atrophy itself; vitamin E, an antioxidant, decreased the degree of atrophy.

It is generally accepted that some transition metals, such as iron and copper, are imphicated in the production of free radicals [3]. We found an increased iron level, especially in microsomes in skeletal muscle atrophied by immobilization, and have suggested the possibility that increased iron may be responsible for the enhanced oxidative stress in atrophied muscle [4]. The role of iron has since been confirmed by the use of deferoxamine, an iron-chelating agent that was shown to suppress the increased oxidative stress [5].

Moreover, we suggested the increased levels of superoxide anions and hydrogen peroxide in the cytoplasm of the atrophied muscle by showing the changes of antioxidant enzyme systems in atrophy [6]. Although they themselves are not so reactive, the hydroxyl radical, the most aggressive of radicals, is known to be generated from them through the catalytic function of iron [3]. Therefore, we suggested that hydroxyl radicals might be formed in atrophy from the increased superoxide anions and hydrogen peroxide in the cytoplasm and the increased iron in microsome [7].

Recently salicylate has been used as a trapping agent in an attempt to detect hydroxyl radicals in vivo [8–11]. When small amounts of salicylate are added into a biological system, its phenolic ring can be attacked by hydroxyl radicals to yield dihydroxybenzoic acids (DHBs) [12], which can then be determined using high-performance liquid chromatography (HPLC) [12]. In the present investigation, to verify our suggestion described above, we measured the in vivo generation of hydroxyl radicals in atrophied muscle using the salicylate method.

2. Materials and methods

2.1. Animals

Twelve male Wistar rats (15 weeks old) were used according to 'Guiding Principles in the Care and Use of Animals'. Under anaesthesia the ankle joint of one hindlimb was immobilized in the fully extended position (i.e. with the soleus muscle in a shortened position), as described previously [2]. The procedure for limb immobilization, as such, had no significant effects [4]. The period of immobilization was 12 days.

Seven rats were injected intraperitoneally with salicylate 12 h before sacrifice. Salicylate was given in the form of sodium salt in a saline solution in a dose of 100 mg/kg b.wt. The other five rats were injected with a saline solution as placebo.

After the 12-day immobilization, soleus, typical slow red muscles from both hindlimbs (atrophied and contralateral) were collected, the contralateral one being used as a control. The water contents of both muscles were the same, and we measured wet tissue weight instead of dry weight. The muscles were stored in liquid nitrogen until sample preparation. All samples were assayed within a week of collection.

2.2. Reagents

Special grades of 2,3-dihydroxybenzoic acid (2,3-DHB) and 2,5-dihydroxybenzoic acid (2,5-DHB) were purchased from Tokyo Kasei

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(Tokyo, Japan). Sodium salicylate was from Nakalai Tesque (Kyoto, Japan). All other reagents were of the best grade available commercially.

2.3. Measurement of 2,3-DHB

The muscle was homogenized with a 5-fold vol. (w/v) of ice-cold 10% trichloroacetic acid under argon gas flow to lessen the oxidative–reductive change. The homogenate was left to stand on ice for 20 min and centrifuged at 12,000 × *g* for 10 min. The supernatant was retained and directly injected into the HPLC system.

The analytical procedure was a modification of the HPLC method of Davis et al. [13]. The HPLC system consisted of a solvent delivery system (Maltisolvent system 600E; Waters), an autoinjector (U6K; Waters), a column inlet filter (0.5 μm × 1.5 mm; Rheodyne), an ultraviolet (UV) absorbance detector (Lambda-Max model 481; Waters), an electrochemical detector (ECD; Amperometer Σ875; IRICA, Japan), and computing integrators (D-2500; Hitachi: M741 data module; Waters). Detection of peaks was at 240 nm for UV detection and at 0.6 V (applied potential) for ECD. The column was an Ultrasphere ODS (250 × 4.6 mm, 5 μm; Beckman), and the mobile phase was 5.0% aqueous acetic acid with 2.0 g/l of ammonium acetate and 5.0 mg/l of EDTA (pH 3.0). The mobile phase was prepared daily, and kept anaerobic by bubbling with helium gas. The flow rate was 1.0 ml/min, and the injected volume was 40 μl. Quantitation was based on peak heights of the ECD detector using external standards.

Our preliminary results showed that the increased iron in atrophied muscles did not change the level of 2,3-DHB. We added 30 μg/g wet tissue of Fe^{2+} (which corresponded to the increased iron in atrophied muscle) to the homogenate of control soleus muscle and determined the level of 2,3-DHB. Consequently, this was not changed.

2.4. Statistics

The data were expressed as mean ± S.E.M. A paired *t*-test was used for the comparison between atrophied and contralateral (control) muscles.

3. Results

The weight of soleus muscles varied from rat to rat, and so we calculated the ratio of atrophied-to-contralateral muscle weight. The ratios were 48.6 ± 1.4 and 48.3 ± 0.7% in rats with salicylate and placebo injection, respectively, and there was no significant difference in the degree of atrophy. Thus, salicylate had no effect on the progress of muscle atrophy.

Representative chromatograms of ECD demonstrating detection of 2,3-DHB, one of the main reaction products formed by the attack of hydroxyl radicals on salicylate, are presented in Fig. 1. As shown in Fig. 1A, our HPLC system could clearly separate 2,3-DHB from 2,5-DHB, the other reaction product. The identity of peaks was determined by co-elution with an authentic standard and by comparison of the voltammogram with an authentic standard. As a result, one clearly separated peak was postulated as the 2,3-DHB peak in the chromatogram of the muscle from the rat injected with salicylate, as demonstrated in Fig. 1B. In the chromatogram of the muscle from the rat injected with placebo, this peak was absent (refer to Fig. 1C). Therefore, this postulated peak in Fig. 1B was identified as 2,3-DHB. By contrast, our HPLC system could not completely separate the 2,5-DHB peak in atrophied muscles: an unknown peak overlapped with that of 2,5-DHB, although it was very small compared with that of 2,5-DHB.

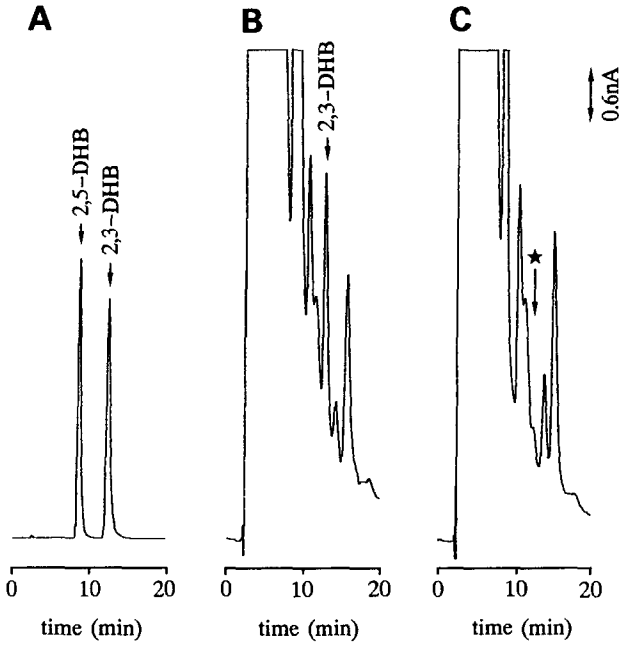

Fig. 1. Representative HPLC-ECD chromatogram demonstrating detection of 2,3-dihydroxybenzoic acid (2,3-DHB) in 12-day atrophied soleus muscle. (A) Authentic standards for 2,5- and 2,3-DHB: the 2,3-DHB peak is clearly separated from that of 2,5-DHB. (B) Extract of 12-day atrophied soleus from a rat injected with salicylate. The chromatogram demonstrated a clear peak of 2,3-DHB; the 2,5-DHB peak could not be separated. (C) Extract of 12-day atrophied soleus from a rat injected with placebo. The 2,3-DHB peak was absent (indicated by a star).

The concentrations of 2,3-DHB were calculated using the peak height of ECD, and are presented in Fig. 2. The concentration of 2,3-DHB in the atrophied muscle was significantly higher than that of control muscle; its level increased more than four times during the 12-day atrophy.

4. Discussion

The hydroxyl radical is one of the most reactive radicals and is thought to be frequently generated in biological systems. Although many methods have been developed to measure it, the available methods with specificity are electron spin resonance-spin trapping and aromatic hydroxylation [12]. With the former is difficult to measure the level of hydroxyl radicals in the in vivo system, since there is no proper spin trap. Alternatively, formation of hydroxylated aromatic products is thought to be a favored reaction pathway under physiological conditions, and so formation of such products can be an index of hydroxyl radical generation in vivo. In particular, salicylate has been used the most of the aromatic products; attack of hydroxyl radical on salicylate generates 2,3-DHB and 2,5-DHB as major products [8–11]. To measure DHBs in biological samples, recent investiga-

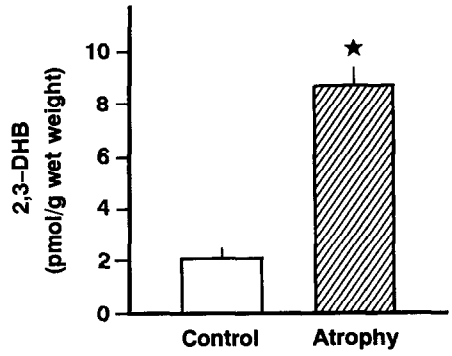

Fig. 2. Levels of 2,3-dihydroxybenzoic acid (2,3-DHB) in 12-day atrophied and contralateral (control) soleus muscles from rats injected with salicylate. Data are mean ± S.E.M. ($n = 7$). *Significant difference at $P < 0.05$ compared with control.

tors have come to use HPLC systems with UV detector or ECD [8–15]. The levels of DHBs in the in vivo system were very low, probably because of their leakage from the cell. Hence, we used ECD detection in the quantitation of DHBs, since ECD detection is reported to be 1,000-times more sensitive than UV detection [14]. In fact, it was very difficult to detect DHBs, especially 2,3-DHB, using UV detector in our experiments.

The reliability of the salicylate method is dependent upon the specificity of DHBs for hydroxyl radicals. The group of Halliwell [15] demonstrated that 2,5-DHB could be produced by the cytochrome P-450 system in mammals by showing that microsomal fractions from mammals treated with inducers of cytochrome P-450 can produce 2,5-DHB but not 2,3-DHB, and, thus, they concluded that measurement of 2,5-DHB could give very misleading results. They recommended that 2,3-DHB is likely to be the main product of interest when using salicylate as a detector of hydroxyl radical generation, because 2,3-DHB was detected only when hydroxyl radical formation was permitted [16]. According to their suggestion, we intended to investigate the change in the 2,3-DHB levels.

The salicylate-injection protocol such as ours has also been reported to maintain salicylate in tissues at a constant level, which was enough to trap hydroxyl radicals [9,17]. Taking these and the above discussions into consideration, the 2,3-DHB level in our experiments is thought to reflect the in vivo production of hydroxyl radicals. Therefore, the increase in 2,3-DHB in the present investigation strongly suggests the accelerating generation in vivo of hydroxyl radicals in skeletal muscle atrophied by immobilization. As far as we know, this is the first report demonstrating in the in vivo system the production of active oxygen species in atrophied muscle.

Previously we suggested the increased generation of superoxide anion in the cytoplasm of atrophied muscle but not in the mitochondria by showing increased Cu-Zn-containing superoxide dismutase (Cu,Zn-SOD) and decreased Mn-containing superoxide dismutase [6], and reported the increased activity of superoxide-producing xanthine oxidase as the source of superoxide anion in the cytoplasm [7]. Enhanced production of superoxide anions and increased Cu,Zn-SOD activity suggested the enhanced production of hydrogen peroxide in the cytoplasm. Due to the unchanged activities of Se-dependent glutathione peroxidase and catalase, which degrade hydrogen peroxide, the level of hydrogen peroxide was expected to be elevated in the cytoplasm of atrophied muscle [6]. This expectation was confirmed by the cytochemical study of hydrogen peroxide using short-term organ culture [7]. We reported increased microsomal iron in atrophied muscle [4] and since proved the important role of iron in oxidative stress [5]. It is generally known that hydroxyl radicals are generated from superoxide anions and hydrogen peroxide in the presence of transition metals such as iron [3]. In view of the simultaneous increase in microsomal iron and the increase in superoxide anions and hydrogen peroxide in the cytoplasm, it was suggested that the generation of hydroxyl radicals might increase in atropied muscle [7]. The present investigation has confirmed this suggestion in the in vivo system and, consequently, strongly supports the mechanism of oxidative stress in muscle atrophy which has been presented in a series of our work.

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