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Hydroxyl Radical Generation and Lipid Peroxidation in C_2C_{12} Myotube Treated with Iodoacetate and Cyanide

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To mimic exercise-induced events such as energetic impairment, free radical generation, and lipid peroxidation *in vitro*, mouse-derived C_2C_{12} myotubes were submitted to the inhibition of glycolytic and/or oxidative metabolism with 1 mM iodoacetate (IAA) and/or 2 mM sodium cyanide (CN), respectively, under 5% CO_2/95% air up to 180 min. Electron spin resonance (ESR) analysis with a spin-trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) revealed time-course increases in spin adducts from hydroxyl radical (DMPO-OH) and carbon-centered radical (DMPO-R) in the supernatant of C_2C_{12} myotubes treated with the combination of IAA + CN. In this condition, malondialdehyde (MDA) and lactate dehydrogenase (LDH) were released into the supernatant. By the addition of iron-chelating 1 mM deferoxamine to the C_2C_{12} preparation with IAA + CN, both ESR signals of DMPO-OH and DMPO-R were completely abolished, and the release of MDA and LDH were significantly reduced, while cyanide-resistant manganese superoxide dismutase had neglegible effects on these parameters. Hence, a part of the injury of C_2C_{12} myotube under IAA + CN was considered to result from the lipid peroxidation, which was induced by hydroxyl radical generated from iron-catalyzed systems such as the Fenton-type reaction. This *in vitro* model would be a helpful tool for investigating the free radical-related muscle injury.

Keywords: C_2C_{12} myocyte, iodoacetate, sodium cyanide, ESR, malondialdehyde, cell death

INTRODUCTION

Exhaustive exercise causes an imbalance between energetic utilization and generation in working skeletal muscles. Decreased mitochondrial respiratory control[1] and degradation of high-energy phosphates[2] were demonstrated in exercised muscles, indicating the energetic impairment. Such exercise is also known to induce the overgeneration of oxygen free radicals in

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muscles,[1,3,4] which can lead to loss of membrane integrity and cellular functions *via* peroxidation of polyunsaturated lipids.[1,5–7] Levels of lipid peroxidation products, measured as conjugated dienes and/or thiobarbituric acid-reactive substances (TBARS), were elevated in the skeletal muscle[1,8] and urine[8,9] after exercise *in vivo*.

Recently, for simulating exercise-induced events in myocytes, experimental models with chemical metabolic inhibitors have been utilized instead of submitting muscles to exercise. For instance, intra-aortic injection of *ortho*-iodosobenzoic acid, an inhibitor of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), induced histopathological changes in rat hindlimb muscles similar to those in exercise-induced rhabdomyolysis. Additionally, several *in vitro* studies have also employed the chemical inhibition of glycolytic and/or mitochondrial enzymes to mimic biological changes caused by the energetic impairment. In various sorts of non-myogenic cell culture, depleted adenosine 5′-triphosphate,[10–12] oxygen free radical generation[13–15] and membrane injury[13–15] were demonstrated.

In the present study, we made an attempt to apply the metabolic inhibition to cultured skeletal myocytes to evaluate the ability of an *in vitro* model reflecting the free radical-mediated muscle injury. To reproduce the substrate depletion and mitochondrial dysfunction, mouse-derived C_2C_{12} myotubes were treated with 1 mM iodoacetic acid (IAA) and/or 2 mM sodium cyanide (CN), inhibitors of GAPDH and cytochrome oxidase, respectively, in presence of molecular oxygen. We here detail generating free radicals in the preparations of C_2C_{12} myotubes by using the electron spin resonance (ESR) method with a spin-trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), and also illustrate the release of MDA from C_2C_{12} myotubes during the metabolic inhibition, which indicates membrane lipid peroxidation. The relationship between free radical-mediated lipid peroxidation and cell injury is discussed.

MATERIALS AND METHODS

Materials

DMPO was purchased from Aldrich (Milwaukee, WI, USA), purified by the method of Rosen and Rauckman[16] using Norit A charcoal (Nakarai Tesque, Tokyo, Japan), and stored at −80°C until use. Dulbecco's modified Eagle medium (D-MEM) was obtained from GIBCO (Gland Island, NY, USA) and fetal calf serum (FCS) was purchased from JRH (Lenexa, KS, USA). Butylated hydroxytoluene (BHT; 2,6-Di-*t*-butyl-*p*-cresol) was from Sigma (St. Louis, MO, USA). Other reagents were of analytical grade from Wako Pure Chemical (Osaka, Japan).

Cell Culture

Mouse-derived C_2C_{12} myoblasts[17] (passage number 31–45) were maintained in D-MEM supplemented with 10% FCS at 37°C under an atmosphere of 5% CO_2 and 95% air. C_2C_{12} myotubes were prepared in 6-well culture plates (Nunc, Naperville, IL, USA) by culturing confluent myoblasts in D-MEM with 2% FCS. Myotubes between 7- and 9-day old were used for all experiments. The cell density was $2.00 \pm 0.09 \times 10^6$ nuclei/well (mean ± SD, $n = 21$) and over 95% of nuclei were in myotubes.

Metabolic Inhibition

The myotube was washed 3 times with Hank's balanced saline solution (HBSS; 1.3 mM $CaCl_2$, 5.0 mM KCl, 0.3 mM KH_2PO_4, 0.8 mM $MgSO_4$, 138 mM NaCl, 4.0 mM $NaHCO_3$, 0.3 mM Na_2HPO_4 and 5.6 mM D-glucose) containing 10 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) at pH 7.4, and pre-incubated for 1 h in 0.4 mL of the same buffer at 37°C under 5% CO_2/95% air. The metabolic inhibition was achieved by adding a 0.1 mL of the same HEPES-HBSS containing 5 mM IAA and/or 10 mM NaCN (final concentrations were

1 and 2 mM, respectively) and incubating myotubes at 37°C under 5% CO_2/95% air up to 180 min.

Spin-trapping and ESR Analysis

In the spin-trapping experiments, pre-incubation of C_2C_{12} myotube was performed in the presence of 40 mM DMPO. Then metabolic inhibition was started by adding IAA and/or CN as described above. The culture supernatant was withdrawn into a flat quartz cell (60 × 10 × 0.3 mm inner size) and placed in the cavity of a JEOL Model RE-1X ESR spectrometer. All ESR spectra were recorded at room temperature and the spectrometer settings were: magnetic field 334.6 ± 5 mT, sweep time 8 min, modulation frequency 100 kHz, modulation amplitude 0.2 mT, reciever gain 2.5×10^3, time constant 0.3 s, microwave frequency 9.415 GHz, and microwave power 14 mW.

Measurement of Supernatant MDA

For the quantification of MDA released into supernatants, a high-performance liquid chromatographic (HPLC) method[18] was employed. This method is based on the pre-column TBA reaction followed by the separation of red TBA adducts. For the derivatization, 200 µl of the culture supernatant, 300 µl of 0.5% aqueous TBA solution and 10 µl of 0.8% BHT (dissolved in glacial acetic acid) were mixed in a 2-ml screw-capped plastic tube, incubated at 95°C for 20 min and cooled in tap water. Then 300 µl of chloroform was added to the mixture. After vigorous mixing and centrifugation (1000× *g*, 3 min), the aqueous phase (upper layer) was collected, filtrated by a 0.22 µm membrane filter (Spartan 13, Schleicher & Schuell, GmBH) and subjected to the following HPLC. For separating TBA adducts, a HPLC system equipped with a Model L-6200 pump (Hitachi), a Model 7125 syringe-loading sample injector (Rheodyne, CA, USA), a Model CTO-2A column oven (Shimadzu, Kyoto, Japan), a Model 490E variable-wavelength detector (Waters, Milford, MA, USA) and a Model D-2500 chromatography integrator (Hitachi) was employed. An isocratic elution of 40 mM acetate buffer (pH 5.5) : methanol (6 : 4) was performed at a flow rate of 0.8 ml/min on a Senshu-pak Pegasil ODS column (4.6 × 250 mm; SSC, Tokyo, Japan) maintained at 35°C. Fifty microliter of the sample was injected and the eluent was monitored at 532 nm (0.04 AUFS) for 10 min. To provide calibration for the TBA-MDA adduct, a set of standards (125–1000 pmol/ml 1,1,3,3-tetramethoxypropane) was included in each batch of derivatization.

Evaluation of Cell Injury

Lactate dehydrogenase (LDH) activities in supernatants were analyzed for evaluating the plasma membrane injury by a Model 736-20 automatic analyzer (Hitachi) with a commercial assay reagents (Wako).

Statistical Analysis

All statistical analyses were performed using Student's *t*-tests for unpaired data.

RESULTS

Figure 1 shows typical ESR spectra of culture supernatants collected from C_2C_{12} preparations with 1 mM IAA and/or 2 mM CN in the presence of spin-trap 40 mM DMPO. While neither IAA nor CN caused detectable change in ESR spectra throughout the observation period, the combination of IAA + CN induced the rise of ESR signals. These signals were identified with a complex of a 1 : 2 : 2 : 1 quartet signal ($A_N = A_H = 1.50$ mT) and a 1 : 1 : 1 : 1 : 1 : 1 sextet signal ($A_N = 1.57$ mT, $A_H = 2.29$ mT), indicative of DMPO-OH and DMPO adducts from carbon-centered radicals (DMPO-R), respectively.[19] Time-course increase in both DMPO-OH and DMPO-R (Figures 1 and 2) clearly indicates that corresponding free radicals were consistently produced in the C_2C_{12} culture

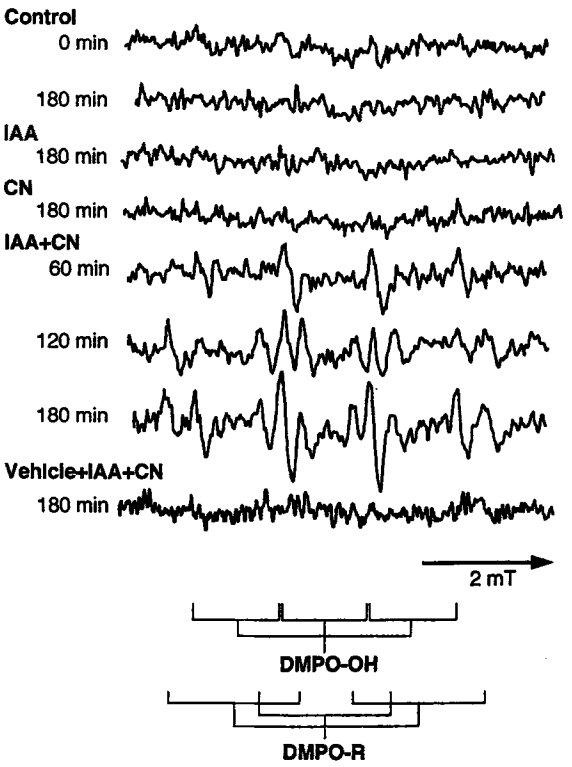

FIGURE 1 Typical ESR spectra of supernatants of C_2C_{12} myotubes treated with 1 mM IAA and/or 2 mM CN in presence of 40 mM DMPO up to 180 min. Each spectrum was recorded by single scan with the constant analytical condition as described in Materials and Methods. Detected signals in the IAA + CN preparation consisted of a 1:2:2:1 quartet signal with hyperfine coupling constants $A_N = A_H = 1.50$ mT, and a sextet signal with $A_N = 1.57$ and $A_H = 2.29$ mT, indicative of DMPO-OH and DMPO-R adducts, respectively.

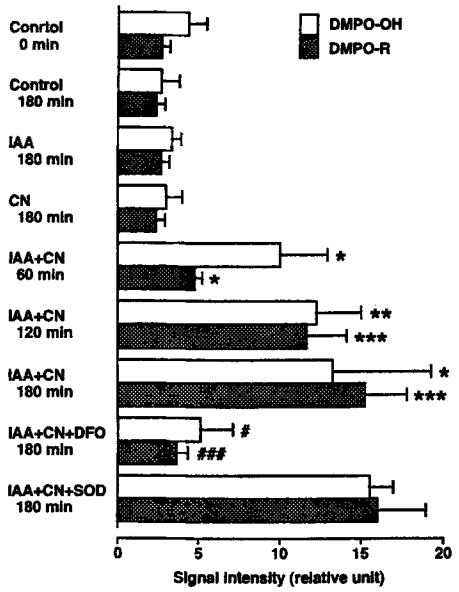

FIGURE 2 Relative signal intensity of DMPO-OH and DMPO-R detected in supernatants of C_2C_{12} myotubes treated with IAA and/or CN up to 180 min. Each point represents the mean ± standard deviation of 3–6 independent experiments. *, ** and *** significantly different from the untreated control with $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. # and ### significantly different from IAA+ CN with $p < 0.05$ and $p < 0.001$, respectively.

system. For characterizing the generating pathway of detected free radicals, antioxidants were co-incubated in the C_2C_{12} preparation with IAA + CN (Figure 2). Iron-chelating 1 mM deferoxamine (DFO) significantly abolished both DMPO-OH and DMPO-R signals at 180 min ($p < 0.05$ and $p < 0.001$, respectively), while CN-resistant 10 U/ml manganese superoxide dismutase (Mn-SOD) had no obvious effect on the signal intensity. Catalase, commonly used in ESR experiments for catalyzing hydrogen peroxide (H_2O_2), could not be applied to the present study because of its susceptibility to cyanide.[20]

MDA released into the supernatant were measured by the HPLC method[18] for the evaluation of oxidative degradation in cellular lipids. The combined treatment with IAA + CN induced the time-course elevation of supernatant MDA concentration, while neither IAA nor CN did when used alone (Figure 3). The addition of 40 mM DMPO (used as a non-specific radical-scavenger) or 1 mM DFO significantly (approx. 50–70%, $p < 0.001$) supressed the MDA release caused by IAA + CN. While the former was more effective on MDA supression, no additive effect was observed when DMPO and DFO were simultaneously used. On the other hand, 10 U/ml Mn-SOD tended to enhance the increasing MDA.

The LDH release from myotube was analyzed to evaluate the plasma membrane injury. The supernatant LDH activity was gradually increased when the myotube was treated with the

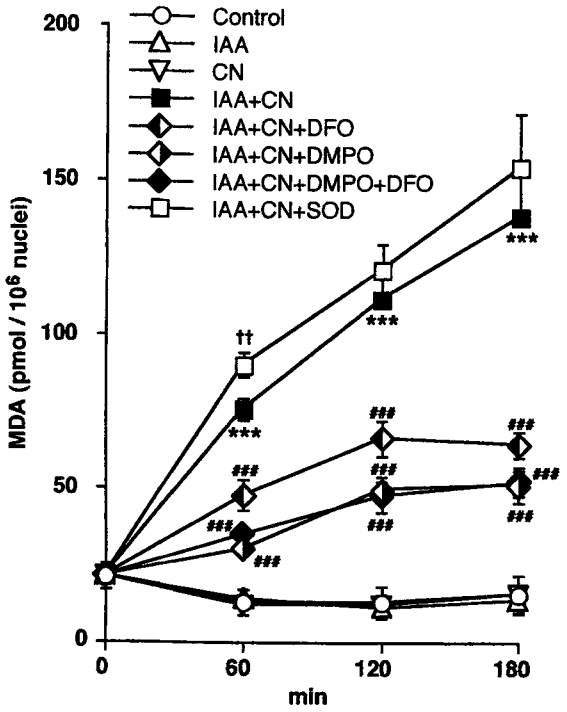

FIGURE 3 Time course changes in the supernatant malondialdehyde concentration. Each point represents the mean ± standard deviation of 3–6 independent experiments. *** significantly different from the untreated control with $p < 0.001$. †† significantly increased from IAA + CN with $p < 0.01$. ### significantly decreased from IAA + CN with $p < 0.001$.

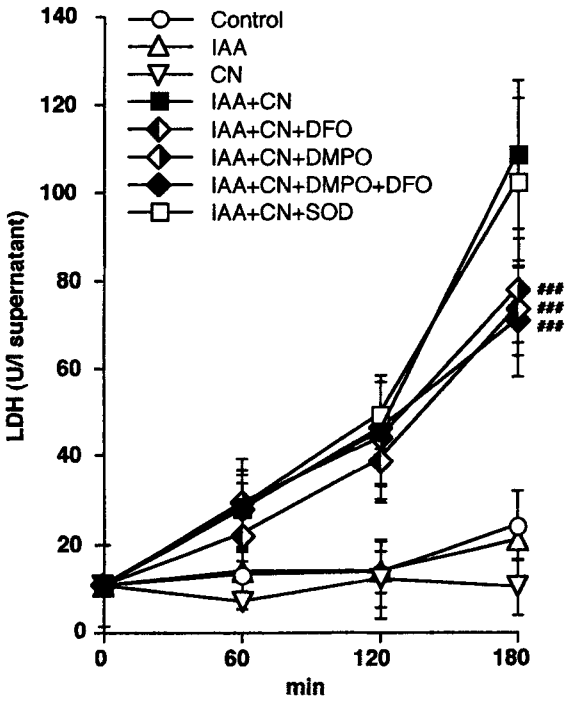

FIGURE 4 Release of LDH from C_2C_{12} myotubes treated with IAA and/or CN up to 180 min. Each point represents the mean ± standard deviation of 12–18 independent experiments. ### significantly decreased from IAA + CN with $p < 0.001$.

combination of IAA + CN (Figure 4). Neither IAA nor CN had significant effect on LDH release during the observation period when used alone. Addition of 40 mM DMPO and/or 1 mM DFO showed cytoprotective effects on C_2C_{12} myotubes treated with IAA + CN at 180 min, while 10 U/ml Mn-SOD did not. There was no additive cytoprotective effect of DMPO + DFO.

DISCUSSION

In vitro chemical metabolic inhibition has been utilized to simulate the biological changes caused by energetic impairment. Several articles[13–15] have shown the endogenous oxygen free radical generation in different sorts of cells treated with IAA and/or CN, however, there still remained uncertainty regarding the identification of radicals. By using the spin-trapping ESR technique, we clearly demonstrated that the metabolic inhibition with 1 mM IAA and 2 mM CN induced the increase in DMPO-OH and DMPO-R adducts in the C_2C_{12} culture supernatant.

Previous reports[21–23] described that the presence of $^{\bullet}OH$ was able to be verified by detecting $R^{\bullet}$ (carbon-centered radicals such as alkyl radicals), since $^{\bullet}OH$ posesses high reactivity among oxygen free radicals and readily causes hydrogen abstraction from carbohydrates. In our study, both DMPO-OH and DMPO-R signals were effectively abolished by the 1 mM ferric iron chelator DFO in the supernatant, indicating that the DMPO-OH in the IAA + CN preparation in a major way corresponded to the direct trapping of $^{\bullet}OH$, which rised from the Fenton-type reaction

catalyzed by the redox-active iron. Additionally, since DFO has been known to work as an extracellular chelator,[24] the observation in our spin-trapping experiments might mainly reflect extracellular events as follows:

$$H_2O_2 + Fe^{2+} \rightarrow {}^{\bullet}OH + OH^- + Fe^{3+}$$

$${}^{\bullet}OH + DMPO \rightarrow DMPO\text{–}OH$$

$${}^{\bullet}OH + R\text{–}H \rightarrow H_2O + R^{\bullet}$$

$$R^{\bullet} + DMPO \rightarrow DMPO\text{–}R$$

In this report we do not directly address the intracellular source of H_2O_2 which is required for $^{\bullet}OH$ generation. The O_2^--generating xanthine oxidase should be inhibited by CN.[25,26] Dawson *et al.*[15] submitted rat hepatocytes to the chemical metabolic inhibition with IAA + CN and demonstrated that oxygen free radicals were generated from the complex III (ubiquinol-cytochrome *c* oxidoreductase) of mitochondrial respiratory chain. Boveris[27] treated isolated bovine heart mitochondria with CN and showed the H_2O_2 generation, which was indicative of that a sufficient Mn-SOD activity was naturally present in the matrix to convert generating O_2^- into H_2O_2. In our ESR experiments, the exogenous Mn-SOD had minimal effect on the ESR spectra of IAA + CN preparation. This result supports that the most of generated O_2^- radicals had been already converted into H_2O_2 by the mitochondrial Mn-SOD before the trapping by DMPO.

On the other hand, it must be noted that the overgeneration of $^{\bullet}OH$ could also result from the loss of enzymatic antioxidative defences. Catalase[20] and glutathione peroxidase,[28] both of which are major H_2O_2-catalyzing enzymes, are inhibited by IAA and/or CN, suggesting that H_2O_2 might readily accumulate in the C_2C_{12} culture system and contribute to the iron-catalyzed $^{\bullet}OH$ production.

For monitoring membrane lipid peroxidation, experiments were performed to measure MDA released into the supernatant. Previous *in vitro* studies revealed that MDA were accumulated in culture media in various oxidative conditions,[29–31] instead of its high reactivity to cellular components.[32] Our data clearly demonstrates that the treatment with IAA + CN, which induced $^{\bullet}OH$ production, resulted in the significant MDA release from C_2C_{12} myotubes. In mammalian tissue samples, major precursors of MDA are considered to be peroxidized arachidonic acid and docosahexaenoic acid (reviewed in [32]). The $^{\bullet}OH$ from Fenton-type reaction is an well-known initiator of lipid peroxidation.[33] Another initiator, iron–oxygen complex, has also been shown to abstract hydrogen atoms from polyunsaturated fatty acids in absence of $^{\bullet}OH$.[34] In the present study, since the $^{\bullet}OH$ scavenger DMPO and/or iron-chelating DFO effectively decreased MDA generation, we speculate that the lipid peroxidation was in a major way initiated by $^{\bullet}OH$. In addition, the exogenous Mn-SOD tended to enhance the MDA production from IAA + CN-treated myotubes (approx. 10%). This result suggests the ability of that part of a generated O_2^- was converted into H_2O_2 by the supernatant Mn-SOD and contributed to the following $^{\bullet}OH$ production and lipid peroxidation, although O_2^- was not directly detected in the ESR study as described above.

On the other hand, we were not able to obtain data regarding the possible propagation and degradation of lipid peroxides which result in the MDA generation. In our ESR experiments, neither peroxy ($ROO^{\bullet}$) nor alkoxyl ($RO^{\bullet}$) radical, which are required in the propagation, was detected. We have made attempt to measure phosphatidylcholine hydroperoxides (PC-OOH), one of the molecular species of peroxidized membrane phospholipids, and found that the PC-OOH concentration was less than 20 fmol/10^6 nuclei throughout experiments (data not shown). Further study is required to elucidate the total kinetics of lipid peroxidation and degradation in this model.

Numerous mechanisms have been proposed to explain cell injuries during *in vitro* chemical metabolic inhibition with iodoacetate and/or cyanide.

Loss of high-energy phosphates,[11] reduced mitochondrial membrane potential,[15] lack of intracellular calcium homeostasis[11–13,35,36] and free radical-induced membrane degradation[13–15] have been believed to be significant. In the C_2C_{12} preparation with IAA + CN, the $^{\bullet}OH$ scavenger 40 mM DMPO and iron-chelating 1 mM DFO showed evident cytoprotective effects, indicating that iron-catalyzed $^{\bullet}OH$ generation and subsequent lipid peroxidation had deleterious roles in cell injury, at least in part. Hence, in conclusion, the present *in vitro* model would be helpful for investigating the free radical-mediated injury of polynuclear myocytes in such as exercise-induced myopathies.

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