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Purification, identification, characterization, and cDNA cloning of a high molecular weight extracellular superoxide dismutase of hamster that transiently increases in plasma during arousal from hibernation

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Abstract

We previously studied antioxidant profiles in the plasma of hibernating Syrian hamsters and found a transient increase of a superoxide radical-scavenging activity during the arousal phase. In this report, we purified and identified the high molecular weight superoxide dismutase (SOD)-like factor from the plasma of arousing hamsters. The cyanide-sensitive 240 kDa SOD-like factor showed a significant homology to mammalian extracellular SOD (EC-SOD) reported, although the molecular mass of EC-SOD was 135 kDa. The cDNA cloning revealed that the 240 kDa SOD-like factor was identical to the hamster ortholog of EC-SOD. It consisted of 245 amino acid residues including a signal sequence of 20 amino acid residues. Five cysteine residues that would participate in inner- and inter-subunit bonds were well conserved among species. Interestingly, there were four potential *N*-glycosylation sites in hamster EC-SOD, whereas there is only one site in other species. The amino acid sequence analysis indicated that three of the four sites were modified. These results suggest that the anomalistically high molecular weight of hamster EC-SOD is ascribed, at least in part, to the addition of extra sugar chains. Furthermore, results obtained here also propose the involvement of EC-SOD in the antioxidative defense of hibernating hamsters.

Keywords: cDNA cloning; EC-SOD; Hibernation; Multimer formation; *N*-glycosylation; Plasma; Purification; Syrian hamster

1. Introduction

When Syrian hamsters (*Mesocricetus auratus*) are kept under the conditions of cold and short photoperiod, most animals go into the state of hibernation (Hoffman, 1968; Newcomer et al., 1987; Arai et al., 2005). Hibernation is characterized by the profound decrease in body temperature and metabolism, which is thought to promote survival during periods of severe climate and food shortage (Lyman et al., 1978). The torpor, a quiescent state with low body temperature, of Syrian hamsters lasts normally 2 to 5 days. The prolonged torpor bouts are interrupted by periodic arousals, during which physiological parameters of hibernators restore rapidly to near-normal levels (Carey et al., 2003). Hibernating animals, in consequences, experience repeated cycles of torpor and arousal throughout the hibernation.

The core body temperature of torpid hamsters was kept slightly above the ambient temperature and the metabolic rate fell to a few to several percents compared with that of euthermic ones (Hoffman, 1968; Newcomer et al., 1987). Physiological parameters such as respiratory and blood flow rates and heart beats were significantly depressed throughout the torpor (Hoffman, 1968; Newcomer et al., 1987; Osborne and Hashimoto, 2003; Osborne et al., 2005). On the other hand, the rewarming phase was accomplished within only a few hours and was accompanied by a massive heat production in brown adipose tissue (BAT) and a rapid recovery of blood flow and metabolism (Osborne and Hashimoto, 2003). The profound elevation of oxygen consumption, surged up to 300% of euthermic level during the arousal phase, was reported in hamsters (Osborne and Hashimoto, 2003) as well as some other mammalian hibernators (Cranford, 1983;

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Tøien et al., 2001). The augmented aerobic respiration leads to the large burst in mitochondrial respiration, followed by an elevated generation of reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($_{\bullet}OH$) (Chevion et al., 2003). The rapid elevation of ROS concentration may exceed the limits of normal antioxidant defense system during arousal phase (Carey et al., 2003), and if that were the case, it would cause irreversible oxidative damages to the cell membrane, lipid molecules, and DNA in the nucleus as well (Bergamini et al., 2004). However, hibernators seem to tolerate repeated cycles of torpor and arousal (Lyman et al., 1978; Newcomer et al., 1987; Carey et al., 2003). In this respect, it is likely that hibernators have acquired a specific or powerful defense system against the oxidative stress, which is expected to emerge during the arousal phase from torpor.

In the previous report, we investigated a variation of antioxidative capacity in the plasma of hibernating hamsters. We found a transient increase of a high molecular weight (HMw) superoxide dismutase (SOD)-like activity in the plasma at the mid- to late-arousal phase (Okamoto et al., 2006). Among many antioxidants, SOD plays important roles in antioxidative defense by converting a superoxide radical to less toxic hydrogen peroxide and oxygen (Fattman et al., 2003). In plasma, the 135 kDa extracellular SOD (EC-SOD/SOD3) is known to be the predominant SOD isozyme in most mammalian species (Marklund, 1984a; Oury et al., 1996; Fattman et al., 2000; Petersen et al., 2004). However, the apparent molecular mass of the HMw SOD-like activity in the hamster plasma was 240–260 kDa. Some other antioxidants found in plasma such as Cu, Zn-SOD/SOD1, Mn-SOD/SOD2, ceruloplasmin, glutathione, ascorbate, α-tocopherol, and albumin-bound bilirubins are also known to scavenge superoxide radical; even so, they do not show such a high molecular weight (Goldstein et al., 1979; Wu et al., 1991; Kretzschmar and Muller, 1993; Sies and Stahl, 1995). Recently, an octameric form of human EC-SOD was reported (Due et al., 2006). Though it showed an apparent molecular mass of about 270 kDa, it was estimated to represent only 1% of the activity in the purified material.

To understand the antioxidant profile in the plasma of hibernating hamsters further, we purified and identified the HMw SOD-like factor. The determination of the N-terminal amino acid sequence of the HMw SOD-like factor demonstrated its homology to mammalian EC-SOD with a molecular mass of 135 kDa. At the same time, we cloned the cDNA of hamster EC-SOD and analyzed the purified EC-SOD protein to confirm the relationship between the HMw SOD-like factor and hamster EC-SOD. The results clearly showed that the HMw SOD-like factor is identical to the hamster ortholog of EC-SOD. Some biochemical properties of hamster EC-SOD and its potential roles in the physiology of hibernation are discussed.

2. Materials and methods

2.1. Blood sampling from arousing hamsters

All treatments and surgical procedures were performed in accordance with guidelines set by the institutional animal experiment review board. Syrian hamsters (*Mesocricetus auratus*) were bred in our laboratory under normal breeding conditions (ambient temperature: $T_a = 23 \pm 1$ °C; light:dark photoperiod, L:D = 12:12). Hibernating hamsters were prepared as previously described (Arai et al., 2005; Okamoto et al., 2006). In short, female hamsters were transferred at 19 weeks of age to a cold, short photoperiod environmental room ($T_a = 6 \pm 2$ °C, L:D = 2:22). The hamsters started hibernation at 8–15 weeks after the acclimatization under these conditions. The torpid hamsters (which had experienced more than 3 times of torpor-arousal cycle) were aroused artificially by gentle handling at room temperature. When the rectal temperature reached 32 °C (normally 1.2 h from the onset of the arousal), blood was taken from abdominal aorta with heparin-moistened syringes under anesthesia with pentobarbital. The plasma samples were stored at − 20 °C until use.

2.2. Partial purification of HMw SOD-like factor from the plasma of arousing hamsters

All purification steps were performed at below 10 °C. The 125 mL of plasma collected from 60 arousing hamsters was subjected to the following purification.

2.2.1. DEAE-5PW

The collected plasma was dialyzed against 50 mM Tris–HCl buffer (pH 8.5) and loaded onto a DEAE-5PW anion-exchange column (Tosoh, Tokyo, Japan) equilibrated with the same buffer. Proteins were eluted with a linear gradient of 0–0.2 M NaCl. The fractions containing SOD-like activity (eluted at 15–20 mS/cm) were pooled.

2.2.2. Ammonium sulfate fractionation and phenyl sepharose

The precipitate formed from the pooled fraction with 40–80% saturation of ammonium sulfate was collected and dissolved in 20 mM sodium phosphate buffer (PB, pH 7.0) containing 0.7 M ammonium sulfate. The solution was then loaded onto a Phenyl Sepharose 6B column (Amersham Biosciences, Piscataway, NJ) equilibrated with 20 mM PB (pH 7.0) containing 0.7 M ammonium sulfate. The flowthrough fraction was collected and concentrated with a hollow-fiber ultrafiltration module (pore size: 6 kDa).

2.2.3. Superdex200

The concentrated fraction from the previous step was separated with a Superdex200 column (Amersham Biosciences) equilibrated with phosphate buffered saline (PBS) at pH 7.2. The fractions containing SOD-like activity (*Mr* 180–350 kDa) were pooled and then subjected to N-terminal analysis of the polypeptides.

2.2.4. Purification of EC-SOD from the plasma of heparin-injected hamsters

Two batches of the purification were carried out. Each batch consisted of the plasma collected from 60 hamsters. Sodium heparin (30 IU/body, Novo Nordisk A/S, Denmark) was administrated intravenously into normal euthermic hamsters (body weight: 160–230 g, 18 week-old female) under anesthesia with