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UNUSUAL GENERATION OF HYDROXYL RADICALS IN HEPATIC COPPER-METALLOTHIONEIN OF LEC (LONG-EVANS CINNAMON) RATS IN THE PRESENCE OF HYDROGEN PEROXIDE

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SUMMARY: A mutant strain of LEC rats (Long-Evans rats with a cinnamon-like coat color) develop spontaneous hepatic injury associated with severe jaundice about 4 months after birth. Recently, we obtained evidence which shows an unusual accumulation of copper (Cu) in the liver of LEC rats, followed by the finding of copper-methallothionein (Cu-MT) induction. To know the mechanism for the development of hepatitis in LEC rats, in relation to induced Cu-MT, we examined whether the generation of active oxygen species is observed. When the Cu-MT was treated with H_2O_2, which is formed by dismutation of superoxide anion radicals or NADPH oxidases in living systems, strong ESR signals due to Cu(II) state appeared when measured at 77K. On the same system, ESR signals due to the spin trapped hydroxyl radicals were observed at room temperature when DMPO (5,5-dimethyl-pyrroline-1-oxide) was used as a spin-trapping agent. The present results suggested that Cu-MT of LEC rat has an important pathogenic role by generating hydroxyl radicals, when hydrogen peroxide is produced in cells or tissues.

Recently, a mutant strain of LEC rats (Long-Evans rats with a cinnamon-like coat color) have been found to develop spontaneous hepatic injury associated with severe jaundice about 4 months after birth (1). Genetic analysis of the rats revealed a single autosomal recessive genesis responsible for hepatitis (2). Since the clinical signs of the hepatitis in the LEC rats have been shown to resemble those of human fulminent hepatitis (3), the rat model may be useful for gaining insight into the pathogenesis of the disease. Quite recently, we obtained evidence which shows an unusual accumulation of

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copper (Cu) in the liver of LEC rats, followed by the finding of copper-metallothionein (Cu-MT) induction (4-6). These observations were based on the results of metal determination by neutron activation analysis and atomic absorption spectrometry, gel-filtrations, electron spin resonance (ESR) spectrometry, radioimmunoassay and immunohistochemical staining for MT (4-6). However, the actual mechanism for the development of hepatitis in LEC rats, in relation to induced MT, is still unclear. We show here a possible implication for the development of hepatitis in LEC rats, in which an unusual generation of hydroxyl radicals is observed with hepatic Cu-MT purified from LEC rats in the presence of hydrogen peroxide (H_2O_2), as detected by ESR-spin trapping method (7). In contrast, both hepatic Cu-MT induced in normal Wistar rats and MT I from rabbits when exposed to H_2O_2 generated very small amount of hydroxyl radicals. In living systems, H_2O_2 is formed by dismutation of superoxide anion radicals, which are abundantly formed by several systems such as xanthine-xanthine oxidase, NADPH oxidase and NADPH-dependent cytochrome P-450 and in neutrophiles (8, 9).

MATERIALS AND METHODS

Male LEC rats/otk (10) and control Wistar rats at 4 months of age were kept in pathogen-free conditions and were given laboratory diet CRF-1 (Oriental Yeast, Tokyo, Japan) and tap water *ad libitum*. Control Wistar rats were given daily i.p. injection of $CuCl_2$ (10 mg Cu/kg body weight) for 3 days to induce Cu-MT in the liver. Cu-MT was purified by repeated gel-filtrations (Sephadex G-75, G-25 and Superose 12HR) as reported (4-6). The molecular weight of hepatic Cu-MT thus obtained was estimated to be about 6kD by SDS-PAGE. ESR spectra were recorded with a JEOL RE3X ESR spectrometer (Tokyo, Japan) at liquid nitrogen temperature (-196℃) for detection of Cu(II) state and at room temperature (22℃) for spin-trapping method (7). For typical spin-trapping measurements, a mixture (0.2 ml) of various amounts of MT, H_2O_2 and a spin-trapping agent, DMPO (5,5-dimethyl-pyrroline-1-oxide) in 20mM Tris-HCl buffer (pH 8.0) was trasferred into a quartz ESR flat cell, which was inserted in the cavity of the ESR spectrometer. ESR spectra were recorded with a modulation frequency of 100 KHz, modulation amplitude of 0.63 mT, and microwave power of 5 mW. Recording of spectra was started 1 min after the addition of H_2O_2 with scanning every 2 min thereafter. Other instrumental conditions were as follows : magnetic field of 337±50 mT, amplitude of 630 and response of 0.1 sec. The ESR spectra of DMPO-OH adducts were identified by the hyperfine coupling parameters ($a_\alpha^N = a_\beta^H = 1.49$ mT) (7, 11) and computer simulation.

RESULTS AND DISCUSSION

ESR spectra for hepatic Cu-MT solution of both LEC rats and Wistar rats given $CuCl_2$ were almost silent and signals due to Cu(II) were not detected, even after the sensitivity of the instrument was elevated. When the Cu-MT solutions were treated with H_2O_2, strong ESR signals due to two types of Cu(II) state ($g_\parallel$ (1)=2.276, $g_\parallel$ (2)=2.248, $A_\parallel$ (1)=0.0168 cm^{-1}, $A_\parallel$ (2)=0.0178 cm^{-1}) appeared (Fig. 1), indicating the oxidation of Cu(I) to Cu(II) with addition of H_2O_2 as well as alteration of the coordination structure from CuS_4 to CuN_4 site as judged by ESR parameters for many Cu(II) model complexes (12).

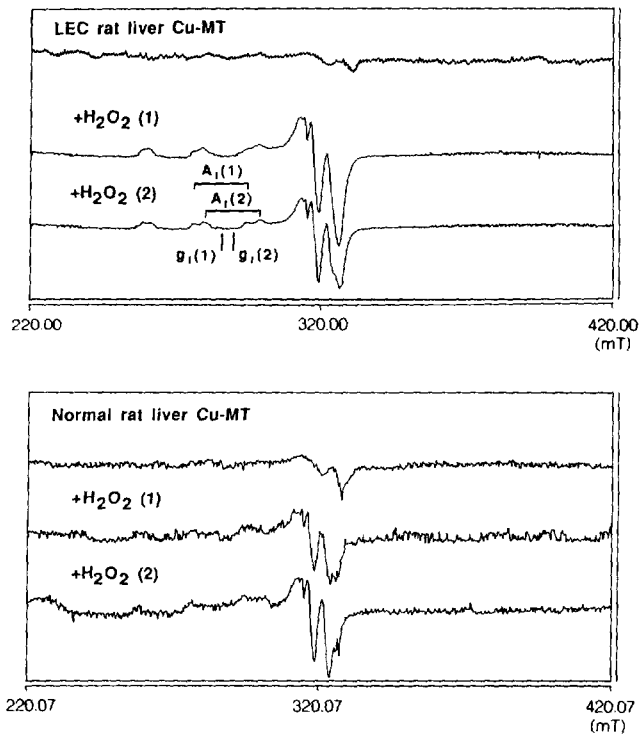

Figure 1. ESR spectra (-196℃) of hepatic Cu-MT in the presence of H_2O_2 at pH 7.4 (20mM, Tris-HCl buffer). Concentrations of Cu in MT were determined by atomic absorption spectrometry after wet-ashing of the samples as 2.76 μg/mg protein for Cu-MT from LEC rats and 1.33 μg/mg protein from normal rats treated with $CuCl_2$. Protein concentrations of hepatic Cu-MT from LEC rats and normal rats treated with $CuCl_2$ in a quartz ESR tube (volume, 0.2ml) were 1.08mg/ml and 0.69mg/ml, respectively. Concentrations of H_2O_2 were 0.82mM for (1) and 1.76mM for (2).

These results demonstrate that Cu in Cu-MT is in a reduced Cu(I) state, in good agreement with finding on Cu-MT isolated from *Neurospora* (13).

The oxidation of Cu(I) to Cu(II) in the presence of H_2O_2 suggests the involvement of the Fenton-like reaction as follows.

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + \cdot OH + OH^- \quad \text{(Fenton reaction) (14)}$$
$$Cu(I) + H_2O_2 \rightarrow Cu(II) + \cdot OH + OH^-$$

Thus we studied whether hydroxyl radicals (· OH) are generated by Cu-MT in the presence of H_2O_2 by the ESR spin-trapping method, which is a most reliable and selective method to detect hydroxyl radicals using a spin-trapping agent like DMPO. Fig. 2 shows the ESR spectrum for spin-trapped hydroxyl radicals in hepatic Cu-MT of LEC rats in the presence of H_2O_2. The spectrum consists of a 1:2:2:1 quartet with splitting of $a_\alpha^N = a_\beta^H = 1.49$ mT, where a_α^N and a_β^H represent the hyperfine splitting of a nitroxyl nitrogen and β-hydrogen atom, respectively. Based on these splitting constants as well as the 1:2:2:1 line shape, the spectrum was assigned to be the DMPO-OH adduct (7, 11). Protein concentration- and time-dependent generations of hydroxyl radicals are

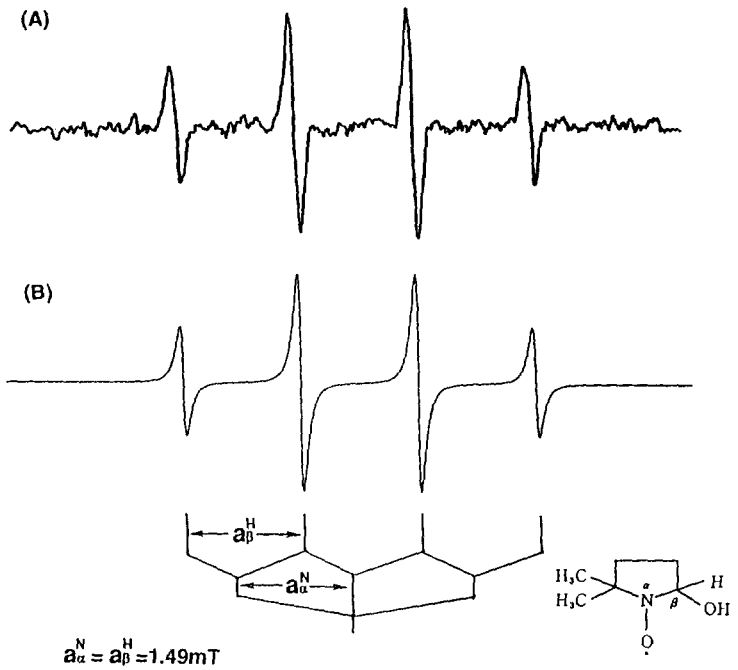

Figure 2. ESR-spin trapping with DMPO in the system of hepatic Cu-MT from LEC rats and H_2O_2 at pH 8.0 (20mM, Tris-HCl buffer). The Cu-MT sample used in the spin-trapping study was the same as for Fig. 1. (A)ESR spectrum for the system of Cu-MT. Concentrations of hepatic Cu-MT from LEC rats, H_2O_2 and DMPO in a quartz ESR flat cell (volume, 2ml) were 0.022mg protein, 0.875mM and 112mM, respectively. (B) Computer simulated ESR spectrum for DMPO-OH adduct.

observable (Fig. 3). In contrast, very small amount of hydroxyl radicals were generated with hepatic Cu-MT from control Wistar rats or MT-I and II from rabbits. These results suggest that there is difference in molecular structure of hepatic Cu-MT obtained from LEC and normal Wistar rats. Thus we are now determing the primary amino acids sequence in hepatic Cu-MT from LEC rats. The elevated capability for generation of hydroxy radicals by hepatic Cu-MT from LEC rats catalyzes the oxidative modification of proteins (15), which may cause pathological states such as jaundice, hepatitis and hepatocellular carcinoma.

Recently, the free radical scavenging effect of MT has become of interest and several lines of evidence supporting this have been reported by researchers (16-20). However, among them Arther et al. reported the stimulation of peroxidation in rat liver microsomes by Cu, Zn-MT and superoxide anions ($O_2^{\cdot}$), in which the release of Zn and rebinding and alteration of the oxidation state of Cu ion, followed by the polymerization of MT, were observed (21). Our present results together with those of Arther et al. suggest that MT, especially Cu-MT may have an important pathogenic role by generating hydroxyl radicals, when active oxygen species such as superoxide anion radicals and hydrogen peroxide are produced in cells or tissues (8, 9, 15). We are now extending our investigations and details will be reported in a sequel manuscript.

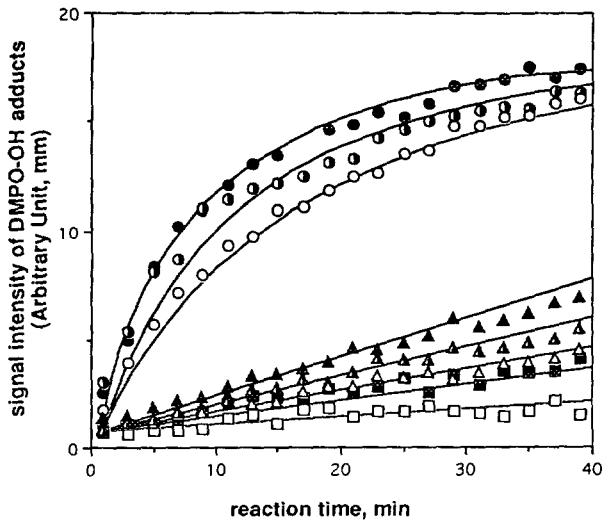

Figure 3. Protein concentration- and reaction time-dependent generations of hydroxyl radicals estimated by ESR spin-trapping with DMPO for the systems of MT and H_2O_2 at pH 8.0 (20mM Tris-HCl buffer). The Cu-MT samples used in the experiments were the same as for Fig. 1. Protein concentrations of hepatic Cu-MT from LEC rats in a quartz ESR flat cell (volume, 0.2 ml) were 0.043mg (●), 0.022mg (◑) and 0.011mg (○). Those of hepatic Cu-MT from normal rats treated with $CuCl_2$ were 0.028mg (▲), 0.014mg (◭) and 0.007mg (△). Those of MT I and II from rabbits were 0.24mg each (■) and (□).

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