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Effect of Heat-treated Ceruloplasmin on the Hepatic Xanthine Oxidase Activity and Type Conversion

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The effect of ceruloplasmin or copper ion on hepatic xanthine oxidase activity and type conversion was investigated using rat liver *in vitro*. It was observed that ceruloplasmin increased xanthine oxidase type conversion depending on duration of its storage. Xanthine oxidase (type O) activity and type conversion in incubation mixture was increased by the addition of heated celuroplasmin in a temperature dependent manner. The type conversion of xanthine oxidase induced by heated ceruloplasmin was returned to normal by the treatment with DTT or penicillamine. The effect of copper ion on type conversion of xanthine oxidase was similar to that of heated ceruloplasmin.

Key words: Ceruloplasmin, Copper ion, Xanthine oxidase, Type conversion

INTRODUCTION

Ceruloplasmin (ferroxidase:Fe(II): oxygen oxidoreductase, EC 1.16.3.1.) is a member of the α-globulin family: it reduces dioxygen to water (Halliwell *et al.*, 1989; Halliwell, 1987). Ceruloplasmin is synthesized in hepatocytes secreted into the blood stream, then it diffuses into cells of different organs such as the heart, kidney and brain (DiMascio *et al.*, 1989; Gutteridge, 1988). This enzyme is involved in copper transport and also influences the transport and storage of iron (Minotti *et al.*, 1987). Ceruloplasmin consists of 1,046 amino acids (Aruoma *et al.*, 1989) and has 3 homologous parts which can be further divided into 2-3 subunits (Ursini *et al.*, 1989). Ceruloplasmin binds 5-8 copper atoms on its 3 different binding sites (Minotti *et al.*, 1987), although ceruloplasmin is capable of binding other transition metal ions (Halliwell *et al.*, 1985). The blue oxidase ceruloplasmin is the major copper-containing protein of human serum. The functional properties of ceruloplasmin *in vitro* have led to suggestions that its role *in vivo* is that of a serum antioxidant or a catalyst of iron mobilization from reticuloendothelial cells by virture of its ferroxidase activity. However, despite a considerable amount of work on the structure and function of this acute phase protein, its true physiological role is unclear. Studies have been hampered by the pronounced lability of ceruloplasmin during isolation and storage. It has been shown that proteolysis is responsible for an artifactural appearance of the subunits reported by others (Ursini *et al.*, 1987). In addition, several reports suggest that physically treated ceruloplasmin is susceptible to various conditions. The products of proteolytic attack and low-intensity UV irradiation have previously been used as a model of oxidative stress on ceruloplasmin. Although it has been the subject of intensive investigation, the effect of oxidative stress on ceruloplasmin is poorly characterized.

Recently, it was reported that during aging ceruloplasmin is subjected to oxidative modifications which are likely to be the source of conformational changes around the copper sites leading to an intramolecular electron rearrangement among the various copper sites (Musci *et al.*, 1993). In a recent study, it was shown that xanthine oxidase is the major source of oxygen radicals which are linked to many diseases (Marx, 1987). In normal conditions, xanthine oxidase exists *in vivo* almost entirely (about 90%) as the dehydrogenase form using NAD^+ as an electron acceptor which dose not produce superoxide anion radical. It is demonstrated that the xanthine dehydrogenase was converted to the xanthine oxidase form which uses molecular oxygen as an electron acceptor producing free radicals in various pathophysiological conditions (Mckelvey *et al.*, 1986; McCord, 1985). In this paper, we have studied the influences of heat-treated ceruloplas-

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min on xanthine oxidase activity and type conversion since it is believed to be the initiative enzyme for the oxidative stress process.

MATERIALS AND METHODS

Chemicals

Bovine serum albumin, ceruloplasmin, dithiothreitol (DTT), penicillamine, xanthine sodium salt and nicotinamide adenine dinucleotide were purchased from Sigma Chemical Co. (St Louis, MO, USA). Other extra pure chemicals were commercially available from local vendors.

Preparation of enzyme source

Male rats weighing 250-280 g (Sprague-Dawley strain) were killed by exsanguination from the abdominal aorta under light ether anesthesia. The liver was exhaustively perfused with ice-cold normal saline through the portal vein until uniformly pale, and immediately removed and weighed. After trimmed and minced, the pieces of liver were homogenized with 4 volume of ice cold 0.1M potassium phosphate buffer (pH 7.4) solution. The homogenate was centrifuged 10,000×g for 20 min. The supernatant was further centrifuged at 105,000×g for 60 min. The resultant cytosolic fraction was used as the enzyme sources of xanthine oxidase.

Xanthine dehydrogenase activity

The xanthine dehydrogenase activity (Stirpe *et al.*, 1969) was assayed by measuring spectrophotometrically the amount of uric acid formed from xanthine sodium with NAD^+ in the incubation mixture.

Xanthine oxidase activity and type conversion

Xanthine oxidase activity (Stirpe *et al.*, 1969) was aerobically determined by measuring the rates of uric acid formation without NAD^+ in the reaction mixture from xanthine sodium as substrate. The reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.5), 0.1 ml of enzyme, 0.06 mM of the substrate and distilled water in a final volume of 4 ml. The reaction was carried out at 37℃ for 15 min. The rate of type conversion from xanthine dehydrogenase (type D) to xanthine oxidase (type O) was represented as type O/D+O.

Protein assay and statistical analysis

Protein content (Lowry *et al.*, 1951) was determined using bovine serum albumin as a standard. The differences between the experimental group were analyzed with the student's t-test.

RESULTS AND DISCUSSION

Type conversion of xanthine oxidase according to the storage duration

It has been suggested that ceruloplasmin is hydrolyzed by biological proteinase, and releases copper ion.

After storage at 4℃ for 6 months and 1 years, ceruloplasmin was tested on xanthine oxidase type conversion in Fig. 1. There was a linear relationship between the type conversion rate of xanthine oxidase and storage period of ceruloplasmin.

This result implies that ceruloplasmin was denaturated and free copper ion release from ceruloplasmin accelerated xanthine oxidase type conversion. Generally, it is well known that the increment of xanthine oxidase (type O) activity significantly generated superoxide anion radical and elevated active oxygen species which induced the various diseases such as atherosclerosis, diabetic mellitus, cancers and aging.

Effect of heat-treated ceruloplasmin on the xanthine oxidase activity and type conversion

Ceruloplasmin contains a high concentration of copper and is denatured by physical treatments such as heating. Therefore, heated ceruloplasmin effects on xanthine oxidase activity and type conversion was investigated. The xanthine oxidase type conversion was not altered by heat treatment up to 50℃, but increased in the heat-treated temperature dependent manner above 60℃ (Fig. 2). As shown in Fig. 3, the xanthine oxidase activity was markedly increased by the addition of heated ceruloplasmin above 60℃ in the reaction mixture, and the increment is the typical pattern of type conversion. On the other hand, xanthine dehydrogenase activity was decreased by the addition

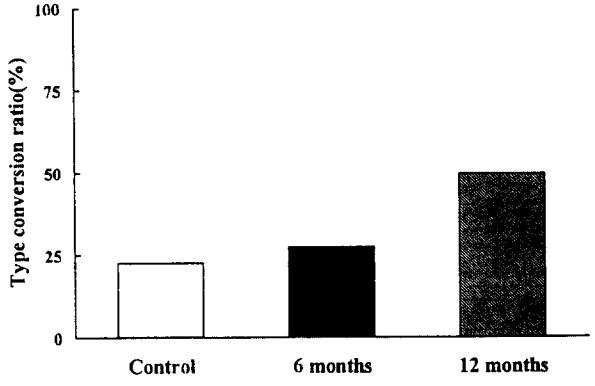

Fig. 1. Effect of storage duration of ceruloplasmin on the typeconversion of xanthine oxidase *in vitro*. The final concentration of ceruloplasmin in incubation mixture was 50 units/ml. The assay procedure was described in the experimental methods. Values are mean for 3 separate experiments.

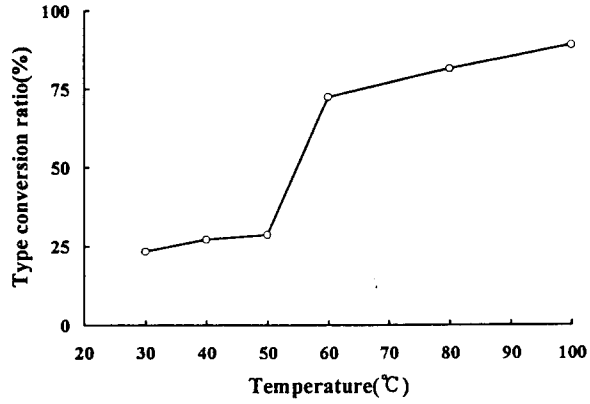

Fig. 2. Effect of heat-treated ceruloplasmin on the type conversion of xanthine oxidase *in vitro*. The final concentration of ceruloplasmin was 50 units/ml. The heating time of ceruloplasmin was 10 minutes. Values are means for 3 separate experiments.

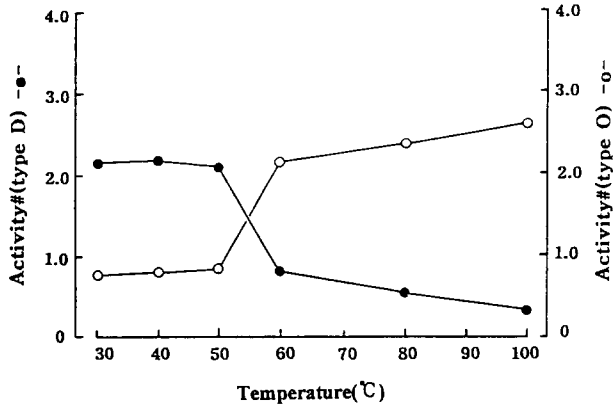

Fig. 3. Effect of heat-treated ceruloplasmin on the hepatic xanthine oxidase (type O) and xanthine dehydrogenase (type D) activity. The final concentration of ceruloplasmin in incubation mixture was 50 units/ml. The heating time of ceruloplasmin was 10 minutes. Unit: uric acid nmoles/mg protein/min

of heated ceruloplasmin. It could be expected that the higher temperature treatment of ceruloplasmin may induce conformational changes in the chemical structure of ceruloplasmin which affect the biological role of ceruloplasmin as an antioxidant protein. The generation of reactive oxygen species such as superoxide anion, hydrogen peroxide and hydroxyl radical by xanthine oxidase is well known. The effect of heated ceruloplasmin on the xanthine oxidase activity has a very important role in the body. These results indicate that the conformational changes of ceruloplasmin may cause differential function of ceruloplasmin according to the pathophysiological role of xanthine oxidase which generates free radicals.

Effect of DTT or penicillamine on heated ceruloplasmin-induced type conversion of xanthine oxidase

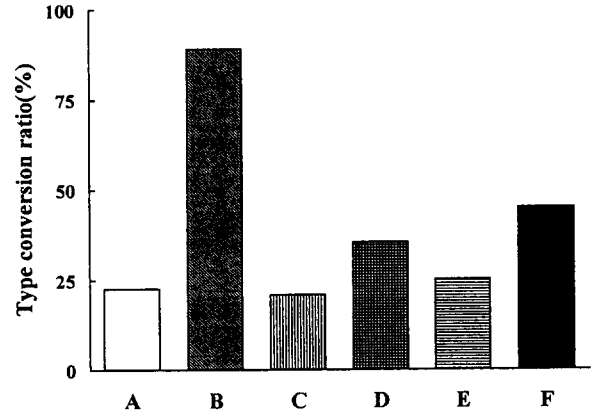

Fig. 4. Effect of penicillamine or DTT on the heated ceruloplasmininduced type conversion of xanthine oxidase. The assay procedure was described in the experimental methods. Values are means for 3 separate experiments. A: control, B: heated ceruloplasmin (50 units/ml) at 100℃, C: penicillamine (10 mM), D: heated ceruloplasmin+penicillamine, E: DTT (10 mM), F: heated ceruloplasmin+DTT

Heated ceruloplasmin increased the type conversion of xanthine oxidase at 100℃. Thus, DTT or phenicillamine effects on the heated ceruloplasmin-induced type conversion of xanthine oxidase was observed (Fig. 4). The addition of heated ceruloplasmin in reaction mixtures significantly increased type conversion compared to the control. But the addition of DTT or penicillamine powerfully inhibited the increment of xanthine oxidase type conversion by heated ceruloplasmin. These results suggest that the releasing copper ion from heated ceruloplasmin was chelated by penicillamine and that the increment of type conversion by copper ion was restored to the control level.

Type conversion of xanthine oxidase according to the copper ion concentration

After incubation with copper ion, the type conversion of xanthine oxidase was increased in a dose dependent manner by the copper-ion addition (Fig. 5). By the addition of over 30 μM copper ion in the assay mixture, xanthine dehydrogenase (type D) was completely converted into xanthine oxidase (type O).

Usually, copper ion is bound to apoprotein of ceruloplasmin in blood. It is accepted that copper ions are released from ceruloplasmin when destruction occurs by pathophysiological condition such as inflammation and infection. Therefore, this result and others indicate that copper ion released from ceruloplasmin under pathological conditions is absorbed by (or chelated) ICF and might influence xanthine oxidase type conversion. This copper effect on xanthine oxidase type coversion is related to copper toxicity in the body.

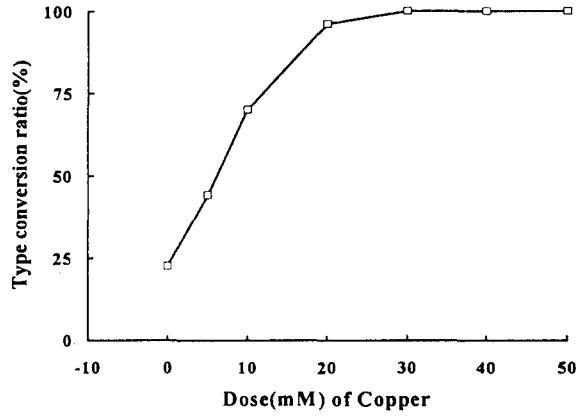

Fig. 5. Effect of copper ion on the type conversion of xanthine oxidase. Values are means for 3 separate experiments.

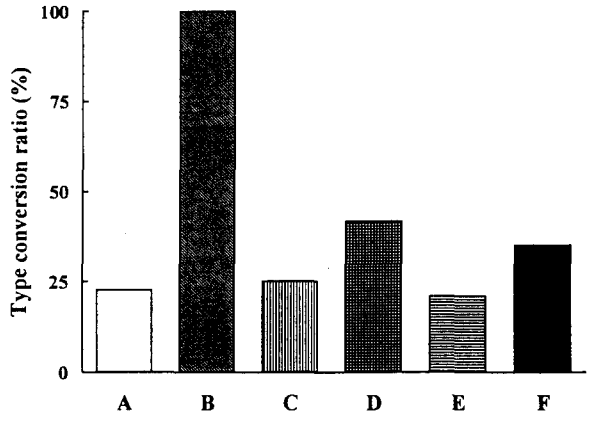

Fig. 6. Effect of dithiothreitol or penicillamine on the copper-inducedtype conversion of xanthine oxidase. Values are means for 3 separate experiments. A: control, B: Cu^{+2} (30 μM), C: DTT (10 mM), D: Cu^{+2}+DTT, E: penicillamine (10 mM), F: Cu^{+2}+penicillamine

Effect of DTT or penicillamine on copper-induced type conversion of xanthine oxidase

Copper ion increased the type conversion of xanthine oxidase, and therefore, DTT or phenicillamine effects on the copper-induced type conversion of xanthine oxidase was investigated (Fig. 6). The addition of DTT or penicillamine markedly inhibited the increment of xanthine oxidase type conversion by copper ion. This effect indicated that penicillamine on xanthine oxidase type conversion by copper ion acts as a direct copper chelator.

It could be suggested that the lability of ceruloplasmin may release copper ion by both heating and oxidative stress. Released free copper ion from ceruloplasmin might induce the type conversion of xanthine oxidase which seems related heavily to the free radical generating process.

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