Research in Pharmaceutical Sciences, April 2008; 3(1): 47-52
Received: Oct 2007
Accepted: Mar 2008

School of Pharmacy & Pharmaceutical Sciences
Isfahan University of Medical Sciences

Original Article

Inhibition of human ceruloplasmin (ferroxidase) by cadmium

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Abstract

Ceruloplasmin (Cp) is a human plasma protein with multiple physiological functions including ferroxidase and oxidase activities. Deficiency or inhibition of the enzyme may lead to some abnormalities, including iron deposition in several tissues and causes various pathological conditions in the body. Direct interaction of cadmium (Cd), a widespread, highly toxic environmental pollutant, with human Cp leads to reversible inhibition of the enzyme. Therefore, investigation of kinetic parameters of Cp in the presence of Cd will lead to a better understanding of mechanism by which Cd inhibit Cp activity. In the present study, Cd inhibited Cp *in vitro* progressively up to a concentration of 2 mM where about 75% of the enzyme activity was lost 10 min after addition of Cd. An inhibitory constant (ki) of about 1.1 mM was calculated from the slope replot. Fluoroscopic study also was carried out on the native and Cd-inhibited enzyme. Maximum emission spectrum of the inhibited enzyme showed an increased level of about 62% with regard to the native enzyme. Cd-induced enzyme inhibition was prevented by sulfhydryl compounds such as glutathione (1.2, 12 mM), and β-mercaptoethanol (12 mM). The data suggest that a conformational change in the native enzyme due to Cd binding caused enzyme inactivation and sulfhydryl groups on the enzyme probably are involved in inhibition by Cd.

Keywords: Enzyme; Ceruloplasmin; Cadmium

INTRODUCTION

Ceruloplasmin (Cp) is a copper containing metalloenzyme found in human blood which is synthesized in liver and carries approximately 95% of total plasma copper (1). The precise physiologic function of Cp is not completely known, but some roles in bactericidal activity, coagulation, iron homeostasis, vascular relaxation, defense against oxidant stress, and lipoprotein oxidation have been proposed for it (2). Cp is an important regulator of iron metabolism *in vitro* and *in vivo*. The specific role of Cp in iron metabolism is not understood, but it may facilitate both cellular iron uptake (3,4) and release (5,6). The role of Cp in iron homeostasis *in vivo* has been confirmed by the discovery of debilitating iron overload in patients with aceruloplasminemia (7). Some investigations have supported the idea that ferroxidation by Cp is a crucial physiological event in iron metabolism (8). In spite of the fact that Cp is involved in iron homeostasis, it seems likely that Cp, due to its unique structure among multicopper oxidases, might be involved in other processes of both enzymatic and nonenzymatic nature.

Cp is an important enzyme in human body and its deficiency or inhibition by toxic agents may lead to iron deposition in several tissues, including brain, liver and pancrease with pathological consequences ranging from diabetes mellitus to dementia (9-11). ρ-phenylen diammonium dichloride (PPD) is a good substrate for determination of the enzyme activity.

Among heavy metals, cadmium (Cd) is of particular concern as an environmental contaminant and its accumulation in human body throughout lifetime is very important (12,13).

Cd affects cell metabolism and catalytic activity of several enzymes. Furthermore, interfering of Cd with the metabolism of some essential metals, such as zinc (Zn), iron,

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calcium (Ca) and copper (Cu), has been well documented (14-16). Copper-dependent lysyl oxidase (17), sheep brain glutathione reductase (18), Cu and Zn-superoxide dismutase in rat liver and kidney (19), δ-aminolevulinate dehydratase from rat lung (20), NADPH-cytochrome P_{450} reductase from leaping mullet (21), human erythrocyte glucose 6-phosphate dehydrogenase (22), Ca^{2+}-ATPase activity of sarcoplasmic reticulum from rabbit muscle (23) and mitochondrial function of aquatic organisms *in vitro* and ATP production (24) have been shown to be inhibited by Cd. Investigation of the Cd effect on Cp, a multifunctional protein with ferroxidase activity, seems to be of special importance, however, little work has been done about the interaction of Cd and Cp activity *in vitro*. Regarding the importance of Cp in metabolism, its inhibition by Cd, may lead to several physiological and biochemical consequences.

Since the mechanism by which Cp is inhibited by Cd *in vitro* is not fully investigated, the aim of this work was to study this process and effects of substances which might decrease or reverse this inhibition and restore normal activity of Cp.

MATERIALS AND METHODS

Materials

Human lyophilized Cp with oxidase activity of 30-50 units per mg protein, PPD, β-mercaptoethanol (BME), glutathione and Cd were obtained from Sigma (U. S. A). All other chemicals were reagent grade.

Enzyme Assay

Cp activity was measured according to the method of Sunderman et al. (25). PPD (8.9 mM) was used as the substrate in 0.1 M acetate buffer with pH 5.4, and the absorbance was measured at 350 nm before calculating the enzyme activity.

Enzyme inhibition

Inhibition of human Cp by Cd was performed by adding different concentrations of the cation to the assay mixture and incubating for 10 min at 25 °C. Lineweaver-Burk plot and the corresponding replot were drawn using linear regression analysis.

Fluoroscopy Measurements

Fluoroscopy measurements were carried out on a Perkin-Elmer LS-3B spectrofluophotometer.

The enzyme (4.8 µg/ml), dissolved in 50 mM tris-HCl buffer (pH 7.4), was incubated in the absence or presence of Cd and Cd plus BME and two different concentrations of glutathione, for 10 min at 25 °C. Samples were excited at 280 nm and the emission spectra were recorded between 300 and 400 nm (26). Protein concentration was determined by the method of Lowry et al. (27).

RESULTS

Cd inhibited human Cp activity progressively up to 2 mM where 75% inhibition was achieved 10 min after addition of Cd (Fig. 1).

Lineweaver-Burk reciprocal plot of Cp in the presence of two fixed concentrations of Cd as an inhibitor is shown in Fig. 2.

It can be seen that the mode of inhibition is noncompetitive. It means that Cd can bind either to the free enzyme form or to the enzyme-substrate complex, producing binary

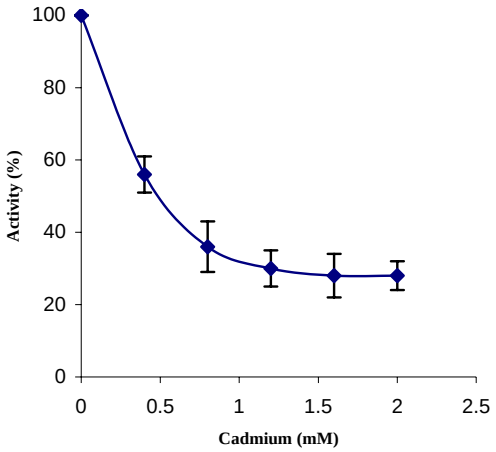

Fig. 1. Inhibition of ceruloplasmin by cadmium. Each point represents the average of two independent experiments.

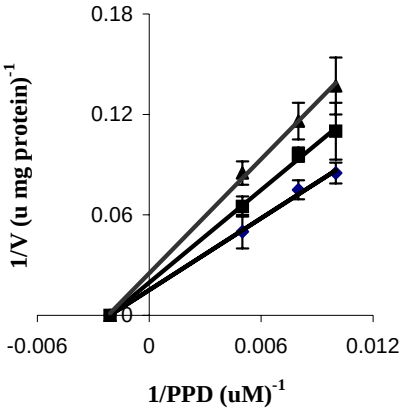

Fig. 2. Lineweaver-Burk reciprocal plot of the ceruloplasmin activity in the presence of Cd as an inhibitor. ρ-phenylendiammonium dichloride (PPD)was the variable substrate. Cd concentrations were zero (◆), 400 µM (■) and 700 µM (▲). Values represent Mean ± SD of three independent experiments.

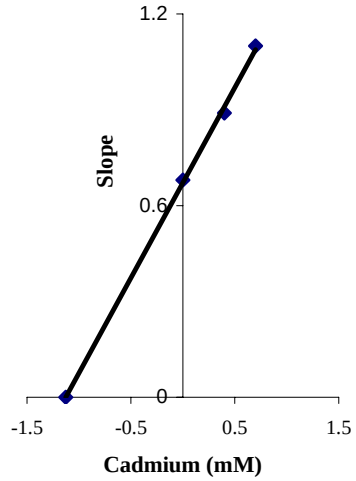

Fig. 3. Replot of inverse slope of the lineweaver-Burk plot vs. Cd concentrations.

and ternary complexes respectively. An inhibitory constant (Ki) of 1.1 mM was calculated from the slop replote (Fig. 3). The enzyme inhibition by Cd was prevented in the presence of sulfhydryl compounds (BME and glutathione). The protection by glutathione was concentration-dependent. (Fig. 4). Maximum emission spectrum of the native enzyme after inhibition by Cd was increased by 62% due to conformational changes caused by Cd binding to the native enzyme. The presence of glutathione or BME reversed the protein fluorescence intensity in a dose-dependent manner, in a way that increasing glutathione concentrations restored the native protein fluorescence (Fig. 5).

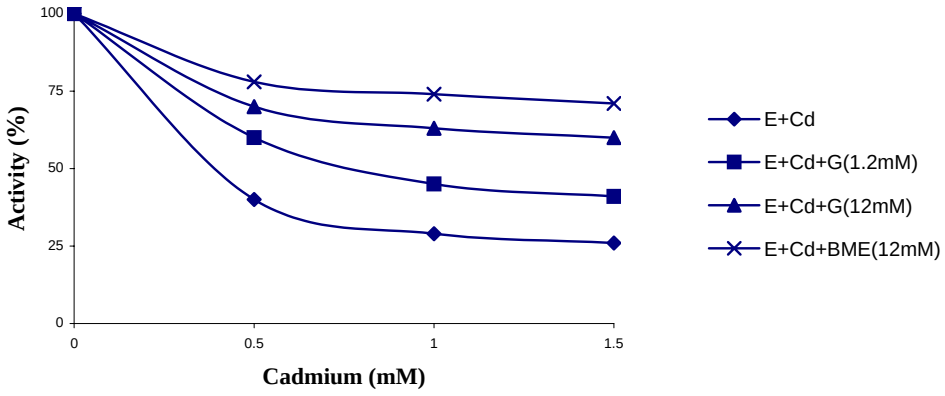

Fig. 4. The effect of different concentrations of Cd on Cp activity in the absence (◆) or presence of 1.2 mM Glutathione (■), 12 mM Glutathione (▲), and 12 mM β-mercaptoethanol (✕). Cadmium chloride was added to the assay mixture and after 10 min the enzyme activity was measured. Each point represents the average of two assays.

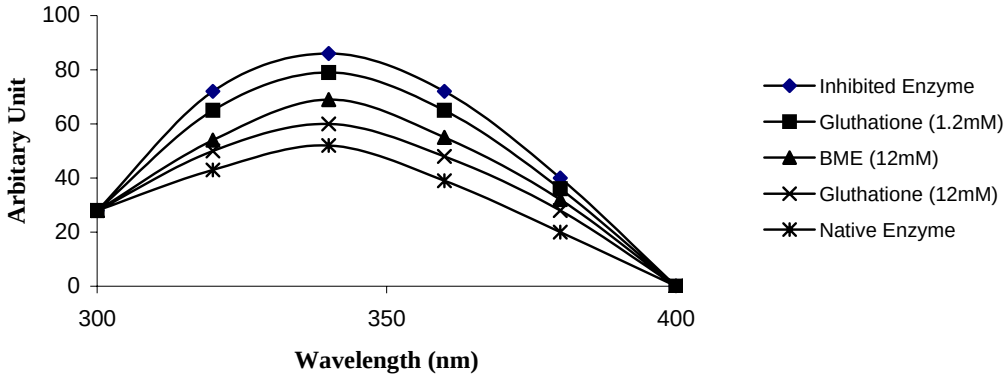

Fig. 5. Fluorescence emission spectra of ceruloplasmin in the absence and presence of Cd and Cd Plus 1.2 mM, 12 mM glutathione and 12 mM β-mercaptoethanol. Samples were excited at 280 nm and the emission spectra were recorded between 300 and 400 nm. The fluorescence intensity is expressed in arbitary units.

DISCUSSION

Several mechaniams have been reported for heavy metals toxicity including inhibitory effects of the metals on enzymes activity. The mechanism of oxidizing activity of Cp is very complex and kinetic investigations of the enzyme in the presence of various ligands can help to clarify this mechanism. The present study was carried out on human Cp in the presence of Cd, a widespread environmental pollutant, which is nonessential and highly toxic trace element in human, in order to determine kinetic parameters of the enzyme.

Data presented here demonstrated that interaction of Cd with human Cp led to reversible inhibition of the enzyme activity. Lineweaver-Burk plot of the kinetic data showed that the mode of inhibition in the presence of Cd is noncompetitive. Binding of Cd to the free enzyme or the enzyme-substrate complex, produces conformational changes in the molecule and therefore, enzyme inactivation. Previous reports indicated that there are high affinities of Cd for binding to sulfhydryl groups of active agents (28). So, Sulfhydryl groups presented in the free enzyme and also in substrate-enzyme complex may be major targets for Cd. For inhibition of ceruloplasmin in the presence of Cd, an inhibitory constant (Ki) of 1.1 mM obtaining from the slop replote calculated. Sulfhydryl compounds protected the enzyme activity against Cd, showing that sulfhydryl groups on the enzyme molecule were involved in the Cd-induced enzyme inhibition. The protection of glutathione against enzyme inhibition was concentration-dependent showing that there is a competition between sulfhydryl group on the glutathione and the sulfhydryl groups on the enzyme molecule for binding to Cd.

The changes observed in Cp fluorescence intensity following inhibition of the enzyme by Cd was also prevented in the presence of compounds containing sulfhydryl groups, providing an evidence for involvment of enzyme's sulfhydryl groups in binding to Cd. Klaassen et al. demonstrated that incorporation of Cd in the sulfhydryl groups of sulfhydryl-rich proteins such as metallothioneines is one of the principal detoxification mechanisms against the metal. They also showed that metallothioneins are intracellular proteins to protect against Cd toxicity (29). The binding of Cd to metallothioneins prevents the free Cd ions from exerting toxic effects. Urani et al. showed that metallothioneines were induced in the human hepatoma cell line $HePG_2$ in a dose-dependent way after incubation of the cells in presence of different concentrations of Cd (30).

Our *in vitro* experiments indicated that Cd inhibits Cp reversibly, and Cd-induced Cp activity inhibition can reduce partially by the addition of substances containing sulfhydryl groups to the medium. On the other hand, as mentioned already, Cp is a multifunctional protein and its activity is increased in stress, infectious diseases, pregnancy, and also during oral contraceptive use (31). However, due to

interference of such factors and the complex nature of Cp, Cd toxicity in human might or might not be inhibited by addition of the above said substances. Therefore, further studies are needed to find whether our *in vitro* results are applicable *in vivo* and to examine possible complications of using sulfhydryl group containing substances in humans.

ACKNOWLEDGMENT

This work was supported by the Research Council of Isfahan University of Medical Sciences. Research No. 385294.

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