
The Antioxidant Defense System Induced in Huh 7 cells by Manganese (II) Administration

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Abstract

The effects of Mn (II) administration on the antioxidant defense system in Huh 7 cells exposed to 5mM MnCl₂ for 12 and 18 hours were investigated. The activities of superoxide dismutase, catalase and glutathione peroxidase were significantly increased in a time dependent manner by exposure to the metal. The activity of glucose-6-phosphate dehydrogenase was decreased after 18 hours and unchanged after 12 hours of manganese treatment. The glutathione reductase was up-regulated after 12 hours and down-regulated by 25% after 18 hours, while the glutathione-S-transferase activities were no changed. Our results indicated that Huh 7 cells developed a specific mechanism against the manganese chloride neutralizing the oxidative stress induced by manganese chloride administration.

Keywords: manganese toxicity, oxidative stress, antioxidant enzyme, Huh 7 cells.

Introduction

Manganese, an essential element in humans and animals, is known to have both a protective and a toxic effect, depending on the concentration. It is a transition element with the capability to exist in several different oxidation states (Mn²⁺, Mn³⁺, Mn⁷⁺), which, as a consequence, confers both powerful prooxidant and antioxidant qualities on this metal [1]. Transition of Mn²⁺ to the trivalent Mn³⁺ state leads to an increased oxidant capacity of the metal which may result in the production of reactive oxygen species (ROS), lipid peroxidation and cell membrane damage [2]. Prolonged Mn (II) exposure induces the formation of reactive oxygen species leading to the impairment of the antioxidant system.

Manganese (II) exhibits various effects in cellular physiology. In trace amounts, it serves as a cofactor for several enzymes. It is an essential component of the mitochondrial superoxide dismutase (Mn - SOD). Complexes between bicarbonate, amino acids, and Mn (II) exhibit catalase - like activity [3,4] and have been shown to protect endothelial cells from H₂O₂ toxicity and from reactive oxygen species produced during oxidative stress. However, long - term exposure to a relatively high concentration of Mn (II) leads to the intracellular accumulation of abnormally high levels [5, 6] that are toxic to a number of cell types, including hepatocytes.

The liver is the main organ involved in the metabolic and detoxification processes and is known to be damaged by chemical pollution and particularly by heavy metals. The hepatocytes were chosen in our experiment due to their important role in the degradation of the xenobiotics.

The common products of xenobiotic metabolism are the reactive oxygen species. Several pollutants perform a part of their toxicity supplying reactive oxygen species as ¹O₂,

$O_2^{\bullet-}$, $\bullet OH$, RO^{\bullet} , ROO^{\bullet} [7]. Oxidative stress appears as a result of xenobiotic action inducing perturbation in antioxidant enzymatic systems.

Reduced glutathione (GSH) is the most abundant cellular thiol, being involved in the protection of cells against the toxic effect of reactive oxygen species and heavy metals [8]. It is capable of complexing and detoxifying heavy metals soon after they enter the cells, thus representing the first line of defense against heavy metals cytotoxicity [9]. Their accumulation in the cells can therefore result in decreased availability of reduced glutathione, due to both GSH oxidation and binding. At the same time, heavy metal such as Cd^{2+} , Hg^{2+} and Pb^{2+} have also been demonstrated to increase the concentration of GSH in the mammalian tissue [10], suggesting that *in vivo* metal treatment could interfere with GSH metabolism.

In this paper, we report the consequence of Mn (II) administration in Huh 7 cells at the level of antioxidant enzymes activities, including catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPX) and glutathione-S-transferase (GST). The investigation of these enzymes is important because they are scavenger for ROS generated in Mn (II) cells exposure. In addition, the activity of glucose-6-phosphate dehydrogenase (G6PD), the enzyme that regenerates the NADPH consumed in the GR and GPX-catalyzed enzymes was also investigated.

Materials and methods

Cell culture. Huh 7 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum and they were incubated at 37°C in the presence of 5mM $MnCl_2$ (concentration which is non cytotoxic) for 12 and 18 hours. The adherent cells were harvested.

Enzymatic assays. The cells were homogenized with 20mM Tris/HCl buffer (pH 7.5), 0.2% Triton X-100, phenylmethylsulfonyl fluoride (PMSF) 0.5mM and sonicated three times for 30 seconds. Total cell lysates was centrifuged at 3000 rpm. for 15 minutes at 4°C. Aliquots of the supernatants were utilized for enzymatic assays.

The CAT activity was assayed by the method of Aebi [11]. The change in absorbance was recorded at 240nm. The CAT activity was calculated in terms of k/ min, where k is the first order rate constant.

The rate of the NADPH formation was a measure of the G6PD activity and it can be followed by means of the increase in extinction at 340 nm [12]. One unit of G6PD activity was expressed as 1 nmol of $NADP^+$ converted in NADPH per minute.

The SOD activity was measured according to the spectrophotometric method of Paoletti et al. [13]. One unit of activity is defined as the amount of enzyme required to inhibit the rate of NADH oxidation of the control by 50%.

The GST was assayed spectrophotometrically at 340 nm by measuring the rate of 1-chloro-2,4-dinitrobenzene (CDNB) conjugation with GSH, according to the method of Haebig et. al. [14] One unit of GST activity was calculated as nmol CDNB conjugate formed/ min, using a molar extinction coefficient of $9.6 \times 10^3 M^{-1} cm^{-1}$.

The GR activity was recorded by the Goldberg and Spooner method [15]. One unit of GR activity was calculated as 1 μmol NADPH oxidised/ min.

The GPX activity was assayed according to the method described by Beutler [16]. The enzyme activity was expressed as U, one unit been the amount of enzyme which oxidized 1 nmol NADPH /min, using a molar extinction coefficient of $6.2 \times 10^3 M^{-1} cm^{-1}$.

All the enzymatic activities were expressed as units per mg of protein. Protein measurements were performed according to the method of Lowry [17], using bovine serum albumin as a standard.

Statistical Analysis. All values were expressed as means \pm SD. The differences between control and manganese-treated groups were compared by Student's t-test using standard statistical packages. The results were considered significant if the *P* value was less than 0.05.

Results and discussion

The transition of Mn^{2+} to Mn^{3+} state leads to an increased oxidant capacity of the metal which may result in the production of reactive oxygen species (ROS) and lipid peroxidation [18]. To determine the response of Huh 7 cells to Mn (II) exposure, the activity of SOD, a primary mitochondria antioxidant enzyme, which catalyses the dismutation of superoxide radicals, was investigated. The activity of SOD substantially increased substantially in Huh 7 cells, after Mn^{2+} exposure. Thus, after 12 hours of 5 mM $MnCl_2$ administration, the increase was 2-fold higher, while, after 18 hours, SOD activity was treble, as compared to the control cells (**Figure 1**).

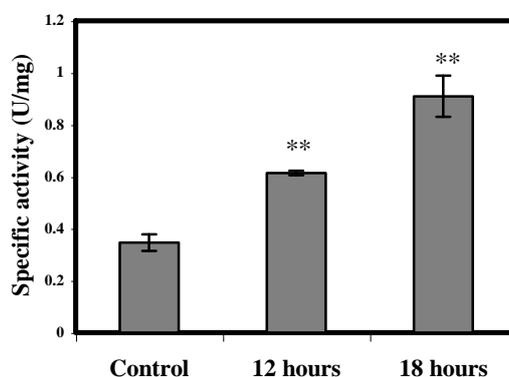


Figure 1. Effect of manganese exposure for 12 and 18 hours on the activity of SOD in Huh 7 cells. Values are means \pm SD. ** *P* < 0.01 vs. control

It has been shown that low concentrations of Mn (II) protect cells against oxidative stress [19]. This protective effect is likely to derive from the fact that Mn (II) can catalyze the dismutation of superoxide radical anion and H_2O_2 under physiological conditions. A number of studies have revealed that most organisms use MnSOD instead of Mn(II) to dismutate superoxide, possibly because: (i) MnSOD dismutates superoxide more effectively than Mn(II); (ii) complexing to SOD protein prevents binding of Mn to other compounds in cells; (iii) under physiological condition, superoxide oxidizes Mn(II) to Mn(III), but Mn(III) is preferentially reduced by other cellular reductants [20, 21]. Our data suggest that cells not only use Mn in the active site of SOD protein to perform catalysis, but also utilize Mn to regulate MnSOD expression. The fact that Mn can induce MnSOD expression was demonstrated in cultured human breast cancer cells and in fish liver [21].

The increase in SOD activity leads to enhancement of hydrogen peroxide (H_2O_2) concentration. Also, once manganese penetrates the cell, it crosses the mitochondria using Ca^{2+} channels, alters the mitochondrial functions and affects the electron transport in the inner mitochondrial membrane. Manganese is sequestered in mitochondria where it inhibits oxidative phosphorylation [22]. As a consequence, the level of H_2O_2 increases and the synthesis and/or release of catalase are stimulated in peroxisomes. Indeed, our experiments

showed that CAT activity increased by 119.2% and 62.3% after 12 and 18 hours of $MnCl_2$ treatment, respectively (**Figure 2**).

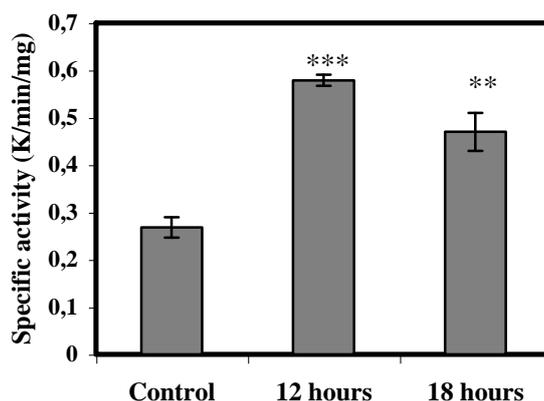


Figure 2. CAT activity variation over 12 and 18 hours in manganese intoxication in Huh 7 cells. Values are means \pm SD. ** $P < 0.01$ vs. control; *** $P < 0.001$ vs. control.

Because that CAT is largely restricted to peroxisomes, most of the cytoplasmatic and mitochondrial peroxides are detoxified by GPX, enzyme that was also investigated. Our results showed that GPX has a more important role in the catabolism of hydrogen peroxide than CAT. Thus, the increase in GPX activity was 6.5-fold higher after 12 hours, and 80-fold higher, after 18 hours of Mn^{2+} exposure, as compared to the control (**Figure 3**). Such an increase in the GPX activities may be due to the higher level of lipid peroxidation induced by manganese as it was reported in Sun and al. works [18]. GPX and GST are enzymes that reduce the level of peroxides protecting the cell from peroxidative damages.

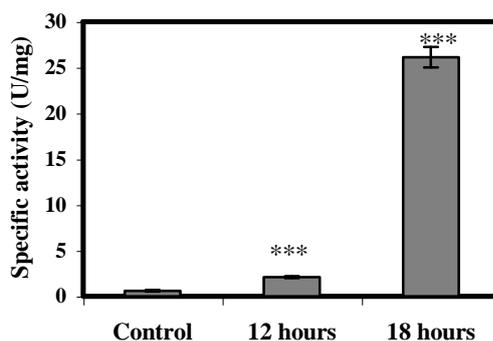


Figure 3. GPX activity in Huh 7 cells of control and manganese exposure for 12 and 18 hours. Values are means \pm SD. *** $P < 0.001$ vs. control.

The activities of the others investigated enzymes: G6PD, GR and GST, are presented in **Table 1**. The activity of G6PD was not changed after 12 hours, while, after 18 hours of manganese administration the activity of this enzyme was decreased by 16.7% (**Table 1**). At high concentration Mg^{2+} and also Mn^{2+} inhibits the Na^+ , K^+ - ATPase, which modifies the Na^+ gradient. As a consequence, the glucose/ Na^+ symport can be impaired and the decrease of the intracellular quantity of this monosaccharide could occur. That's why the rate of the first reaction of the pentose – phosphate shunt is probably decreasing, which may be the cause of inactivation of GPX 18 hours of treatment.

Table 1. Effects of manganese treatment for 12 and 18 hours on the activities of GST, G6PD and GR activities in Huh 7 cells.

<i>Enzyme</i>	<i>Control</i>	<i>12 hours</i>	<i>18 hours</i>
G6PD	0.012±0.0007	0.011±0.0002	0.010±0.0003*
GR	0.012±0.0020	0.018±0.0029*	0.009±0.0007*
GST	0.129±0.0053	0.119±0.0075	0.130±0.0008

Values are means ± SD. **P* < 0.05 vs. control

The GR was up-regulated by 50% after 12 hours and down-regulated by 25% after 18 hours (**Table 1**). GSH cannot cross the cellular membranes, but intracellular pools can be increased by the activity of glutathione reductase [23]. The increase in GR activity, after 12 hours of Mn intoxication may be due to a high cellular need for glutathione, the most abundant cellular thiol, involved in metabolic and transport processes and in protection of cells against the toxic effects of heavy metals. The decreasing of this activity after 18 hours can be correlated to the lower level of G-6-PDH activity at the same time of exposure. If the latter activity is decreased, the quantity of NADPH necessary for GSH regeneration in reaction catalyzed by GR is lower.

There were no changes in GST activities noticed for both periods of manganese administration (**Table 1**). This fact indicated that, GST was not implicated in the reduction of the level of peroxides accumulated in Huh 7 cells after manganese treatment.

Conclusions

Manganese treatment induced the elevation of SOD, CAT and GPX activities in Huh 7 cells, which means that it had generated superoxide and hydrogen peroxide formation.

Our results revealed that Huh 7 cells exposed to manganese (II) chloride developed specific adaptative responses to oxidative stress as it could be noticed from all the analyzed enzymatic specific activity.

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