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Regulation of Heme Oxygenase Activity in Rat Liver during Oxidative Stress Induced by Cobalt Chloride and Mercury Chloride

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Abstract—Activities of heme oxygenase and tryptophan-2,3-dioxygenase and cytochrome P450 content in liver as well as absorption of the Soret band and optical density at 280 nm in serum were determined 2 and 24 h after administration of $HgCl_2$ and $CoCl_2$ and after co-administration of the metal salts with α-tocopherol. Administration of $HgCl_2$ and $CoCl_2$ increased the contents of hemolysis products in the serum, induced heme oxygenase, and decreased cytochrome P450 content in the liver. Injection of $HgCl_2$ increased the activity of tryptophan-2,3-dioxygenase holoenzyme and enzyme saturation with the heme, but administration of $CoCl_2$ decreased these parameters. Pretreatment with α-tocopherol completely blocked the changes induced by $HgCl_2$ after 24 h. Induction of heme oxygenase induced by $CoCl_2$ was not blocked by α-tocopherol, but this antioxidant normalized the increase in the level of hemolysis products in the serum and decrease in tryptophan-2,3-dioxygenase holoenzyme activity and cytochrome P450 content. Mechanisms of regulation of heme oxygenase by mercury and cobalt ions are discussed.

Key words: heme oxygenase, tryptophan-2,3-dioxygenase, mercury chloride, cobalt chloride, α-tocopherol, oxidative stress

Heme oxygenase (EC 1.14.99.3) catalyzes the first rate-limiting step of heme degradation generating carbon monoxide, iron, and biliverdin IXα. The latter is reduced to bilirubin, and this reaction is catalyzed by biliverdin reductase. At least two isoforms of heme oxygenase have been identified; they are the products of separate genes. Both enzyme isoforms are present in the liver, including an inducible (HO-1) and a constitutive (HO-2) form; their ratio is 1 : 2. HO-1 is a stress protein induced by an increase in free heme content in the cell and by factors causing oxidative stress. HO-2 is not induced by the agents influencing HO-1 expression; the main function of this enzyme may be heme binding rather than heme degradation [1]. The induction of HO-1 decreases cellular levels of heme, which is a strong pro-oxidant [2]. Thus, the natural antioxidant bilirubin is accumulated, and this pigment efficiently traps reactive oxygen species [3]. Another product of the heme oxygenase reaction, carbon monoxide, participates in certain regulatory processes, similar to NO [4]. Hence, HO-1 induction can be considered as a constituent of the defense against oxidative damage. Heavy metal ions (like heme) increase HO-1 transcription [5-7] via activation of various transcription factors [8, 9]; however, the regulatory mechanisms are not understood in detail. On the other hand, heavy metal ions and other HO-1 inducers activate free radical oxidation and induce oxidative stress [10, 11]. We have shown that increased level of hemolysis products in serum and their subsequent transport to tissues are mechanisms of the development of oxidative stress during the administration of heavy metal salts [12]. A number of hemoglobin- and heme-binding proteins of the blood and liver limit the pro-oxidant effect of heme, providing for its transport to sites of utilization and degradation [13-16].

Thus, the goal of the present study was to investigate heme oxygenase activity and activities and contents of various heme-containing and heme-binding proteins in liver and serum of rats administered $HgCl_2$ or $CoCl_2$ and co-administered with metal salts and the antioxidant α-tocopherol.

Abbreviations: HO) heme oxygenase; LP) lipid peroxidation; TBA) thiobarbituric acid; MDA) malonic dialdehyde; Co-PP) cobalt-protoporphyrin IX; Tr-2,3-DO) tryptophan-2,3-dioxygenase.

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MATERIALS AND METHODS

Wistar rats (150-180 g) were used in the study. $CoCl_2 \cdot 6H_2O$ and $HgCl_2$ were dissolved in 0.9% NaCl. $HgCl_2$ was injected intraperitoneally (0.7 mg/100 g body weight). $CoCl_2$ was injected subcutaneously (3 mg/100 g body weight). Control animals were injected with the corresponding volumes of 0.9% NaCl. Animals were decapitated under light ether anesthesia 2 and 24 h after metal salt administration. α-Tocopherol acetate was injected intramuscularly (5 mg/100 g body weight) 2 h before metal salt administration. The liver was perfused with cold physiological saline *in situ*.

Heme oxygenase activity was assayed in the liver homogenate [17]. Methemalbumin (final concentration of hemin in the cuvette was 0.033 mM; concentration of human serum albumin was 2.5 μM) was used as the substrate. Samples were incubated in the dark at 37°C for 10 min. Enzyme activity was calculated as the amount of generated bilirubin using extinction coefficient $\varepsilon = 4 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ and expressed in nmol bilirubin/min per mg protein.

Tryptophan-2,3-dioxygenase activity (EC 1.13.11.11) was determined in the liver homogenate by measuring the amount of kynurenine generated from L-tryptophan and expressed in nmol kynurenine/h per mg protein [18]. Activity of Tr-2,3-DO holoenzyme was determined without exogenous hemin, and total enzyme activity was determined in the presence of hemin. Saturation of Tr-2,3-DO with heme was calculated as the ratio of holoenzyme activity to total activity and expressed in percentage [16].

Cytochrome P450 content was determined in the liver homogenate by differential spectrophotometry [19] and expressed in nmol/mg protein (molar extinction coefficient $\varepsilon = 104 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$).

Accumulation of hemolysis products and changes in hemopexin level were determined as the difference in absorption (ΔA) of the serum in the Soret region (390-450 nm) and at 280 nm, respectively, and was expressed in ΔA/mg protein [20, 21].

Haptoglobin content was determined as described [22] and expressed in mg per ml of serum.

The rate of ascorbate-induced lipid peroxidation (LP) in the liver homogenate was determined in medium containing 100 mM Tris-HCl buffer (pH 7.4) and 0.5 mM ascorbate. TBA-reactive products were determined as described [23] and expressed in nmol MDA/mg protein per 10 min incubation (molar extinction coefficient $\varepsilon = 1.56 \cdot 10^5$ $M^{-1} \cdot cm^{-1}$).

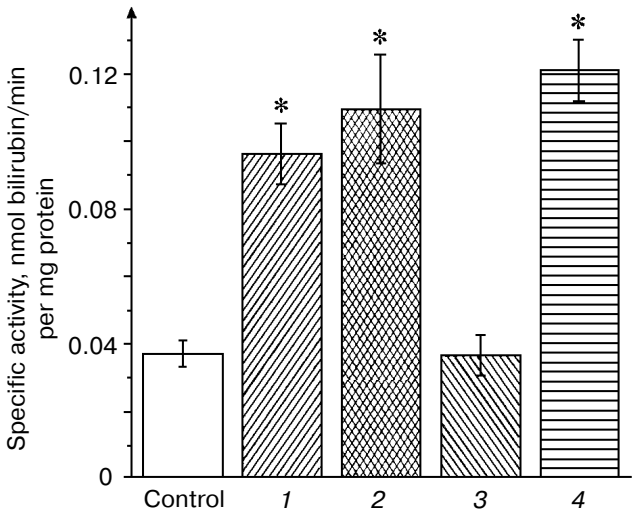
Protein was assayed by the Lowry method as modified by Miller using bovine serum albumin as the standard [24]. Statistical evaluation of the data was performed using the non-parametric Mann–Whitney test [25].

The following reagents were used: Tris, L-tryptophan, and $NADP^+$ from Reanal (Hungary); glucose-6-phosphate and glucose-6-phosphate dehydrogenase from Sigma (USA). Other reagents (of analytical grade) were produced in The Ukraine.

RESULTS AND DISCUSSION

Heme oxygenase activity was increased 24 h after administration of $HgCl_2$ and $CoCl_2$ (figure). Several authors have demonstrated [5, 6] an increase in heme oxygenase activity after administration of heavy metal salts associated with the activation of *de novo* enzyme synthesis. However, it is not known whether heme oxygenase synthesis is induced directly by the metal ions or is mediated by an increase in the free heme levels in the cell. Ions of certain metals (e.g., cobalt) can be incorporated (by ferrochelatase, which catalyzes the last stage of heme synthesis) in protoporphyrin IX, substituting for iron and generating Co-PP, which induces heme oxygenase [5]. On the other hand, other metal ions (including mercury) are not incorporated into protoporphyrin by ferrochelatase but also induce heme oxygenase [26].

The degree of saturation of the cytosolic protein Tr-2,3-DO with heme in liver cells is used as a marker for the level of free heme [5]. In rat liver, Tr-2,3-DO has two forms that include free apoenzyme and apoenzyme bound to heme (holoenzyme); the ration between the forms changes depending on the level of free heme in the cell [5, 16]. In 2 h after $HgCl_2$ administration, holoenzyme activity is increased in rat liver because the saturation of Tr-2,3-DO by heme is increased (Table 1). Total



Heme oxygenase activity in rat liver homogenate 24 h after administration of $HgCl_2$ (*1*) and $CoCl_2$ (*2*) and co-administration of α-tocopherol and $HgCl_2$ (*3*) and of α-tocopherol and $CoCl_2$ (*4*); data are mean ± standard error of the mean; $n = 5$-6; * $p \leq 0.05$ versus control.

enzyme activity remained unchanged. This suggests that increased saturation of the enzyme with heme is due to an increase in the free heme content, and that the levels of apo Tr-2,3-DO are unchanged. During oxidative stress, several sources contribute to the free heme reserve in the liver, including heme of intracellular heme-containing proteins damaged by free radicals and hemoglobin of lysed erythrocytes taken up from the blood stream. When heavy metal ions penetrate into the blood stream, they induce erythrocyte hemolysis [27]. According to the data shown in Table 1, the absorption in the Soret region is increased by 3.5-fold 2 h after $HgCl_2$ administration versus the control, suggesting accumulation of the erythrocyte hemolysis products. In 24 h, this parameter exceeds the control level by 2-fold (Table 2). Hemopexin content in serum decreases during the first hours after $HgCl_2$ injection, and haptoglobin level decreases in 24 h. Decreased levels of the heme- and hemoglobin-binding proteins suggest that administration of $HgCl_2$ induces severe hemolysis. Heme released from lysed erythrocytes is bound by hemopexin and transported by endocytosis into cells containing the hemopexin receptor, including liver parenchymal cells [28]. Binding of the heme–hemopexin complex to the corresponding receptors on the plasma membrane of the liver cells increases the expression of the genes encoding for HO-1 and heme-binding proteins [20, 29]. Increase in the heme oxygenase activity detected 24 h after $HgCl_2$ administration and induction of the heme-binding protein Tr-2,3-DO [30] provide for a decrease in the content of free heme in the case of increased accumulation of hemolysis products in the serum and release of the heme moiety from cytochrome P450. The latter hypothesis is confirmed by decreased contents of cytochrome P450 (Table 2).

Administration of $CoCl_2$ also increased the contents of hemolysis products in the serum, but this increase was significantly less pronounced than that detected in the case of $HgCl_2$ injection (Table 3). This may be due to different

Table 1. Activity and heme saturation of tryptophan-2,3-dioxygenase in liver homogenate and absorption of serum 2 h after $HgCl_2$ administration and co-administration of α-tocopherol and $HgCl_2$ (mean ± standard error of the mean; n = 5-6)

Conditions	Tr-2,3-DO activity, nmol kynurenine/h per mg protein		Saturation of Tr-2,3-DO with heme, %	Absorption, ΔA/mg protein	
	holoenzyme	total		Soret	280 nm
Control	3.62 ± 0.26	9.25 ± 0.54	39.44 ± 3.49	0.023 ± 0.002	0.56 ± 0.02
$HgCl_2$	5.14 ± 0.46*	9.71 ± 0.88	53.65 ± 4.20*	0.081 ± 0.017*	0.42 ± 0.02*
α-Tocopherol and $HgCl_2$	6.79 ± 1.16*	11.79 ± 2.58	60.03 ± 5.09*	0.097 ± 0.01*	0.47 ± 0.03*

Note: The data of Table 1 were obtained during spring and summer and the data of Tables 2-5 were obtained in winter–spring.
* $p \le 0.05$ versus control.

Table 2. Activity and heme saturation of tryptophan-2,3-dioxygenase, content of cytochrome P450 in liver homogenate, level of haptoglobin, and absorption of serum 24 h after $HgCl_2$ administration and co-administration of α-tocopherol and $HgCl_2$ (mean ± standard error of the mean; n = 5-6)

Conditions	Tr-2,3-DO activity, nmol kynurenine/h per mg protein		Saturation of Tr-2,3-DO with heme, %	Cytochrome P450 content, nmol/mg protein	Haptoglobin content, mg/ml	Absorption, ΔA/mg protein	
	holoenzyme	total				Soret	280 nm
Control	5.88 ± 0.50	17.51 ± 1.01	33.62 ± 1.86	0.38 ± 0.02	4.05 ± 0.18	0.033 ± 0.006	0.71 ± 0.04
$HgCl_2$	17.55 ± 2.17*	44.23 ± 1.76*	39.57 ± 4.12	0.23 ± 0.01*	3.29 ± 0.15*	0.066 ± 0.009*	0.75 ± 0.05
α-Tocopherol and $HgCl_2$	7.78 ± 1.02	27.44 ± 3.84*	28.89 ± 2.55	0.35 ± 0.02	3.90 ± 0.14	0.036 ± 0.007	0.80 ± 0.09

* $p \le 0.05$ versus control.

hemolysis mechanisms. Mercury ions can damage erythrocytes directly by interacting with SH-groups of the plasma membrane proteins of these cells [31]. The hemolytic activity of $CoCl_2$ is predominantly associated with the activation of free radical processes by iron ions released during modification of heme and hemoglobin by Co^{2+} [5, 32]. Previously, we demonstrated the presence of Co-PP-containing hemolysis products in the serum of rats injected with $CoCl_2$ [12]. Similar to the heme, Co-PP can form a complex with hemopexin; binding of this complex to the hemopexin receptor induces HO-1, similar to the effect of the heme–hemopexin complex [20]. Binding of heme– or Co-PP–hemopexin with the receptors (but not the penetration of free metalloporphyrins in liver cells) was shown to induce nuclear factor NF-κB transfer from the cytoplasm to the nucleus and activation of c-Jun N-terminal kinase (JNK), which phosphorylates the c-jun protein of the AP-1 complex. The promotor of the HO-1 gene includes binding sites for transcription factors NF-κB and AP-1 [8, 9]; thus, these factors can regulate the expression of this isoenzyme gene. Cobalt ions can penetrate into the liver cells and modify intracellular heme and heme proteins. Significant amounts of Co-PP are accumulated in the liver 16-18 h after administration of $CoCl_2$ to rats [33, 34]. The data of the present work indicate that 24 h after $CoCl_2$ administration, holoenzyme activity, total activity, and heme saturation of Tr-2,3-DO are dramatically decreased, suggesting that the levels of native heme are decreased in liver and Co-PP content is increased (Table 4). Decreased content of cytochrome P450 can be due to degradation of this heme protein as the result of LP activation or to substitution of Co-PP for the heme [35, 36]. Increased contents of free heme or its analog in liver cells induces HO-1. According to Maines [4], formation of reactive oxygen species during interaction of heme with HO-2 is important for this induction; apart from the catalytic site, HO-2 has two additional regulatory heme-binding sites. Unlike heme, Co-PP is not metabolized in the heme oxygenase reaction and is slowly excreted; hence, Co-PP is present for a long time at concentrations sufficient for HO-1 induction in liver cells [5].

Table 3. Activity and heme saturation of tryptophan-2,3-dioxygenase in liver homogenate and absorption of serum 2 h after $CoCl_2$ administration and co-administration of α-tocopherol and $CoCl_2$ (mean ± standard error of the mean; n = 5-6)

Conditions	Tr-2,3-DO activity, nmol kynurenine/h per mg protein		Saturation of Tr-2,3-DO with heme, %	Absorption, ΔA/mg protein	
	holoenzyme	total		Soret	280 nm
Control	5.84 ± 0.84	12.68 ± 0.81	45.66 ± 4.81	0.025 ± 0.004	0.55 ± 0.05
$CoCl_2$	5.83 ± 0.92	14.42 ± 2.47	41.31 ± 2.06	0.044 ± 0.004*	0.48 ± 0.03
α-Tocopherol and $CoCl_2$	6.01 ± 0.58	11.81 ± 0.99	50.99 ± 2.45	0.038 ± 0.009	0.57 ± 0.04

* $p \le 0.05$ versus control.

Table 4. Activity and heme saturation of tryptophan-2,3-dioxygenase, cytochrome P450 content in liver homogenate, and absorption of serum 24 h after $CoCl_2$ administration and co-administration of α-tocopherol and $CoCl_2$ (mean ± standard error of the mean; n = 5-6)

Conditions	Tr-2,3-DO activity, nmol kynurenine/h per mg protein		Saturation of Tr-2,3-DO with heme, %	Cytochrome P450 content, nmol/mg protein	Absorption, ΔA/mg protein	
	holoenzyme	total			Soret	280 nm
Control	5.84 ± 0.84	12.68 ± 0.81	45.66 ± 4.81	0.30 ± 0.02	0.025 ± 0.004	0.55 ± 0.05
$CoCl_2$	1.93 ± 0.26*	9.05 ± 1.09*	22.17 ± 3.28*	0.14 ± 0.01*	0.032 ± 0.016	0.56 ± 0.01
α-Tocopherol and $CoCl_2$	6.08 ± 1.13	24.01 ± 2.34*	25.34 ± 3.86*	0.24 ± 0.02	0.026 ± 0.009	0.61 ± 0.04

* $p \le 0.05$ versus control.

Table 5. Activity of heme oxygenase, activity and heme saturation of tryptophan-2,3-dioxygenase, cytochrome P450 content in liver homogenate, and absorption of serum 4 and 26 h after α-tocopherol administration (mean ± standard error of the mean; n = 5-6)

Conditions	Heme oxygenase activity, nmol bilirubin/min per mg protein	Tr-2,3-DO activity, nmol kynurenine/h per mg protein		Saturation of Tr-2,3-DO with heme, %	Cytochrome P450 content, nmol/mg protein	Absorption, ΔA/mg protein	
		holoenzyme	total			Soret	280 nm
Control	0.037 ± 0.004	5.84 ± 0.84	12.68 ± 0.81	45.66 ± 4.81	0.30 ± 0.02	0.025 ± 0.004	0.55 ± 0.05
α-Tocopherol							
4 h	0.040 ± 0.009	4.29 ± 0.54	12.43 ± 1.04	34.42 ± 2.84*	0.31 ± 0.02	0.026 ± 0.006	0.72 ± 0.1
26 h	0.040 ± 0.004	3.84 ± 1.29	10.87 ± 1.98	33.03 ± 4.88**	0.28 ± 0.02	0.029 ± 0.006	0.50 ± 0.1

* $0.05 \le p \le 0.1$ versus control.
** $p \le 0.05$ versus control.

To study the role of free radical oxidation in the induction of heme oxygenase and decrease in cytochrome P450 levels in animals administered with mercury and cobalt, α-tocopherol was injected 2 h before the heavy metal administration. Pretreatment with the antioxidant completely suppressed $HgCl_2$-dependent induction of HO after 24 h (figure). In 2 h after co-administration of α-tocopherol and $HgCl_2$, increase in hemolysis products and decrease in hemopexin levels in serum were unchanged (Table 1). Hence, activation of free radical-dependent processes is not the main factor inducing erythrocyte hemolysis during the first hours of development of the effects of mercury. However, in 24 h, contents of hemolysis products and haptoglobin in the serum, activity of Tr-2,3-DO holoenzyme, and cytochrome P450 content in the liver did not differ from the corresponding values in the control animals (Table 2). This suggests that extent of erythrocyte hemolysis and the free heme contents in the liver are decreased in 24 h; this may be due to limitation of the pro-oxidant effects of the heme in the case of co-administration of α-tocopherol and $HgCl_2$.

Pretreatment with α-tocopherol did not prevent HO induction by $CoCl_2$ (unlike the α-tocopherol effect on $HgCl_2$-dependent induction). On the other hand, the antioxidant completely suppressed the increase in the rate of ascorbate-induced LP in the liver homogenate 2 h after $CoCl_2$ administration (control: 4.16 ± 0.60; $CoCl_2$: 7.68 ± 1.22, $p \le 0.05$ versus control; α-tocopherol plus $CoCl_2$: 4.94 ± 1.04). Co-administration of the antioxidant and $CoCl_2$ decreased the contents of the hemolysis products in serum during the first hours after metal salt injection (Table 3). In 24 h, the activity of Tr-2,3-DO is not decreased and total activity is increased; hence, decreased saturation of the enzyme with heme is due to increased content of the Tr-2,3-DO apoprotein in the case of co-administration of α-tocopherol and $CoCl_2$ (Table 4). This effect may be associated with the decreased content of Co-PP in the liver or to enhanced secretion of glucocorticoids [37]. Glucocorticoids induce the expression of Tr-2,3-DO [38]. Increased levels of Tr-2,3-DO apoprotein during co-administration of α-tocopherol and $CoCl_2$ suggest that a stress-inducing factor is preserved in the liver, which is not the case when α-tocopherol is co-administered with $HgCl_2$. Suppression by α-tocopherol of a decrease in cytochrome P450 level caused by $CoCl_2$ injection (Table 4) is further evidence suggesting that metal salt-dependent decrease in cytochrome P450 level is mediated by damage to the heme protein caused by free radicals but is not mediated by an increase in heme oxygenase activity.

Injection of the control animals with α-tocopherol did not influence the tested parameters except heme saturation of Tr-2,3-DO, which is decreased (Table 5). Normalization of the contents and activity of the heme-containing proteins of the serum and liver by α-tocopherol may be due to its membrane-stabilizing effect mediated by changes in the physicochemical properties of the membrane (including the erythrocyte membrane) and by inhibition of LP [37].

Thus, $HgCl_2$-dependent HO induction is due to increased levels of free heme in the serum and liver; this is confirmed by an increased amount of hemolysis products and holoenzyme activity and heme saturation of Tr-2,3-DO; on the other hand, $CoCl_2$-dependent HO induction is due to the formation of the heme analog Co-PP. Pretreatment with α-tocopherol prevents HO induction and $HgCl_2$-induced increase in free heme contents in serum and liver. Co-administration of α-tocopherol and $CoCl_2$ decreases the free heme amount (normalizing contents of hemolysis products in the liver and preserving cytochrome P450 levels) and increases the contents of Tr-2,3-DO apoprotein, which is usually associated with glucocorticoid secretion; this suggests the presence of a stress-inducing factor in the liver which can be involved in

HO induction. In general, the extent and duration of heme oxygenase induction by the metal ions are determined by the amount of the substrate and activation of free radical processes as well as by the rates of degradation and excretion of the formed metalloporphyrin complex.

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