Journal of Cellular Biochemistry 57:409–414 (1995)

ARTICLES

Differential Heme Oxygenase Induction by Stannous and Stannic Ions in the Heart

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Abstract Heme oxygenase is the rate-limiting enzyme in heme catabolism, and is induced by oxidative stress, foreign and endogenous chemicals, and many trace elements and heavy metals. This study examined the effect of the oxidative state of the heavy metal tin, on heme oxygenase-1 induction in cardiac tissue. Subcutaneous administration of stannous and stannic chloride failed to induce the enzyme in this tissue. Atomic absorption spectroscopy revealed the absence of tin in the heart cells. Investigation of several metal formulations showed that both stannous and stannic citrate were able to enter the bloodstream from the injection site and into heart tissue. Northern blot analysis revealed that heme oxygenase-1 mRNA was elevated several-fold in rat hearts from animals which received either stannous or stannic citrate, and that mRNA levels corresponded with the increase in enzyme activity. The presence of citrate facilitated the transport of the tin ion into the blood stream and possibly across cardiac cell membrane. The stannous ion was more potent as an inducer of heme oxygenase than was the stannic ion.

Key words: heme, heme oxygenase, mRNA, tin, heart

Heme oxygenase (EC 1.14.99.3) is the rate-limiting enzyme in heme degradation to bile pigments. Expression of the heme oxygenase-1 (HO-1) gene shows marked variations in several cell types [Abraham et al., 1988]. Transcripts are present at high levels in fetal liver which consists mainly of erythropoietic cells, and at much lower levels in adult heart and liver [Abraham et al., 1987, 1988]. Expression of the HO-1 gene is also affected by many environmental agents such as heavy metals [Maines and Kappas, 1977a]. It is believed that the high enzyme activity thus induced may result in a decrease in microsomal heme and cytochrome P450 levels and consequently modulation of important cellular functions [Martasek et al., 1991]. An interesting feature of heme oxygenase induction by heavy metals is the pronounced and sustained effect of the elements on the enzyme. Unlike heme, the natural substrate for the enzyme, heavy metals generally induce HO-1 activity several fold with only a single administration. Moreover, the activity remains elevated for several days before returning to basal levels. The extent of heme oxygenase induction by metal ions, however, varies considerably from one tissue to another [Maines and Kappas, 1977b]. A knowledge of the effect of environmental agents on cardiac tissue is important to an understanding of the effects of environmental agents on cardiac cells, and ultimately, to an understanding of the physiology and pathophysiology of heme proteins in cardiac tissues.

Two heme oxygenase isoenzymes, the products of two distinct genes, have been described [Shibahara et al., 1993; McCoubrey and Maines, 1994]. Heme oxygenase-1 is the inducible form which is distributed ubiquitously in mammalian tissues, whereas heme oxygenase-2 is believed to be constitutively expressed, not inducible by HO-1 inducers and present at high levels of activity in tissues such as brain and testis [McCoubrey and Maines, 1994]. HO-1 is a stress protein and is induced in the heart by hypoxia [Katayose et al., 1993]. A heat shock element has been identified in the 5′-flanking region of the rat HO-1 gene [Okinaga and Shibahara, 1993]. More recently, a binding site for the oxidative stress response factor NF-KB has been located in the human HO-1 promoter region [Lavrovsky et al., 1994].

We examined the oxidative state of the heavy metal tin on cardiac cells because this element

Received July 20, 1994; accepted July 26, 1994.

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displays a significant tissue specific HO-1 inducing activity [Kappas and Maines, 1976]. Our results show that the formulation of the tin as well as its oxidation state are important factors in its ability to induce HO-1.

MATERIALS AND METHODS

Chemicals

Tin (IV) chloride pentahydrate was obtained from Janssen Chimica, (Belgium). Tin Atomic Absorption Standard was obtained from Aldrich (Milwaukee, WI). Heme arginate was obtained from Normosang R (Leiras-Medica, Finland). Zinc deuteroporphyrin bis glycol was obtained from Porphyrin Products (Logan, UT). All other chemicals were obtained from Sigma (St. Louis, MO).

Animal Procedures

This investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No 85-23, revised 1985). Sprague-Dawley (SD) rats weighing approximately 300 g, were obtained from Taconic, and fed and housed under identical conditions. Rats were divided into groups of n = 6, and injected subcutaneously with one of the following: 1 ml saline; 1 ml $SnCl_2$ (500 μM/kg) in saline plus or minus 1,500 μM/kg sodium citrate; 1 ml $SnCl_4$ (500 μM/kg) in saline plus or minus 1,500 μM/kg sodium citrate. All solutions were made immediately prior to injection. The animals were starved and then sacrificed after 4, 16, or 24 h.

Preparation of Microsomes

The animals were sacrificed by decapitation. Organs were perfused with ice-cold saline. Hearts were immediately ground in liquid nitrogen, and then heart and kidney tissues were homogenized in 10 mM tris-HCl, pH 7.5, 0.25 M sucrose. The tissue homogenates were centrifuged for 20 min at 27,000*g* at 4°C. The supernatant was centrifuged at 105,000*g* for 90 min at 4°C. The microsomal pellet was resuspended in 0.1 M potassium phosphate buffer, pH 7.6. Protein concentration was determined by the method of Lowry et al [1951] with bovine serum albumin (Fraction V) as a standard.

Heme Oxygenase Activity

Heme oxygenase activity was assayed by the method of Tenhunen et al. [1969] as modified by Abraham et al. [1985]. Bilirubin levels were determined using a Perkin-Elmer Lambda 7 Spectrophotometer (Perkin-Elmer, Norwalk, CT) using the difference in absorption from 460 to 530 nm. Activity was given as nmol bilirubin produced per h per mg microsomal protein.

Heme Oxygenase mRNA Levels

The animals were sacrificed 4, 5, or 6 h after the last injection, and the organs immediately placed in liquid nitrogen for RNA extraction by the guanidinium isothiocynate/phenol/chloroform method [Chomczysnki and Sacchi, 1987]. The levels of heme oxygenase mRNA were determined by Northern blot analysis of total RNA using cDNA for rat heme oxygenase-1. The probe used in this study was the 883 base-pair EcoR1-Hind fragment of pRHO1, a plasmid containing full-length cDNA for rat spleen heme oxygenase [Shibahara et al., 1985]. This fragment contains the structural gene for all but the first 29 amino acid residues of the enzyme and has been shown to cross-react with heme oxygenase mRNA in rodents [Shibahara et al., 1985]. The DNA probe was labeled according to the primer extension technique of Feinberg and Vogelstein [1983]. Eight micrograms of total RNA from each tissue was electrophoresed on gels containing 1% agarose and 1 M formaldehyde, transferred to nitrocellulose and hybridized with the ^{32}P-labeled probe. The relative amounts of heme oxygenase mRNA present were obtained from the counting of excised filters corresponding to the positive signals of about 18S. The size markers were rat and bacterial ribosomal RNA. Autoradiography was performed for varying lengths of time at −80°C using Kodak XAR-5 film (Kodak, Rochester, NY) with DuPont Lighting Plus intensifying screens (DuPont, Wilmington, DE).

Atomic Absorption

Perfused organs were homogenized (3 ml 10 mM tris-HCl, pH 7.5, 0.25 M sucrose/g tissue), diluted 1:1 with 100% nitric acid and incubated overnight at 45°C. Blood samples were collected in heparinized tubes, diluted with nitric acid 1:1, and incubated as above. Homogenate (50 μl) was diluted with 100 μl 1% triton-X-100, 5 μl matrix modifier (0.2% magnesium nitrate hexahydrate, 2.0% ammonium dihydrogen phosphate, 20% nitric acid), and 845 μl water. Samples were analyzed in a Perkin-Elmer 5000 Atomic Absorption Spectrophotometer, against

a standard curve created from a 1 ppm Tin Standard, and at a wavelength of 286.4 nm.

Analysis of Tin Valency

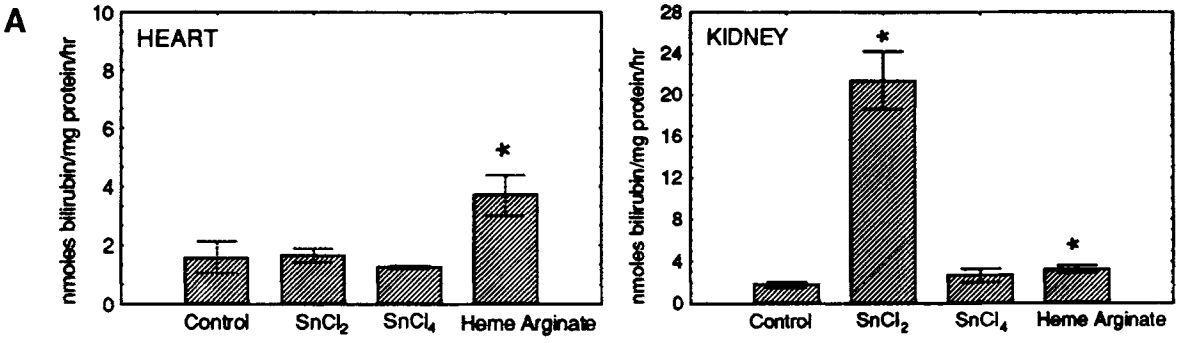
To 10 μl of sample, 10 ml of 0.1 M HCl and two drops of toluene-3,4-dithiol suspension (0.102 g/ml in 0.2 N NaOH) was added. After 5 min the absorbance was read at 540 nm. Sn^{2+} reacts to form the red compound tin (II) dithiolate, whereas Sn^{4+} does not [Cheng et al., 1992]. Sn^{2+} is readily oxidized to Sn^{4+} so all tin solutions were monitored by the above reaction to ensure the valency of the tin injected.

Statistical Analysis

Statistical significance was determined by analysis of variance (ANOVA). The null hypothesis was rejected if the P value was less than 0.05, as calculated, using Newman-Keuls for multiple group comparisons. For comparison of two groups an unpaired t-test was used.

RESULTS AND DISCUSSION

The inorganic metals $SnCl_2$ and $SnCl_4$ were administered to animals by dissolving the metal compounds in saline and immediately injecting subcutaneously. Following 16 h, the heart and kidneys were removed and heme oxygenase activity assessed. As can be seen from Figure 1A, the basal level of heme oxygenase activity in the heart was 1.58 ± 0.54 nmol bilirubin/mg protein/h. Following heme arginate administration the activity increased by 235% to 3.70 ± 0.99. In contrast, neither $SnCl_2$ nor $SnCl_4$ was able to increase heme oxygenase activity in the heart; $SnCl_2$, but not $SnCl_4$ induced HO-1 in the kidney, as expected. As can be seen in Figure 1B, heme oxygenase mRNA was substantially induced in the heart by heme arginate, with or without the heme oxygenase inhibitor zinc deuteroporphyrin IX 2,4 bis glycol (ZnDPBG). However, neither $SnCl_2$ nor $SnCl_4$ influenced heme oxygenase mRNA (Fig. 1B). The lack of effect of the tin on induction of heme oxygenase in the heart, led us to examine the tissue correlation of tin in heart tissue and in the bloodstream following injection. As can be seen in Figure 2A, neither of the tin compounds could be detected in heart tissue. Figure 2B indicates levels of the two forms of tin over time; Sn^{2+} was clearly absorbed from the injection site while Sn^{4+} was not. The absence of Sn^{4+} in the bloodstream



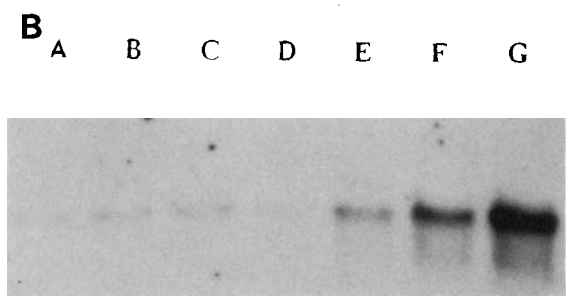


Fig. 1. **A:** Heme oxygenase activities in the heart and kidney ± standard deviation, 16 h after treatment with saline, $SnCl_2$ (500 μmol/kg in saline), $SnCl_4$ (500 μmol/kg in saline), or heme arginate (30 mg/kg). Heme oxygenase activity was determined as discussed in Materials and Methods. *P < 0.05. **B:** Northern blot analysis of heme oxygenase mRNA in the heart. Lane A: saline (4 h); lane B: $SnCl_2$ (500 μmol/kg in saline), 4 h; lane C: $SnCl_2$ (500 μmol/kg in saline), 5 h; lane D: $SnCl_2$ (500 μmol/kg in saline), 6 h; lane E: heme arginate (30 mg/kg), 4 h; lane F: heme arginate (30 mg/kg) + zinc deuteroporphyrin bis glycol (10 μmol/kg), 4 h; lane G: heme arginate (30 mg/kg) + zinc deuteroporphyrin bis glycol (10 μmol/kg), 6 h. Heme oxygenase mRNA was extracted as discussed in Methods. Levels of heme oxygenase mRNA were determined by Northern blot analysis of total RNA using cDNA for rat heme oxygenase-1.

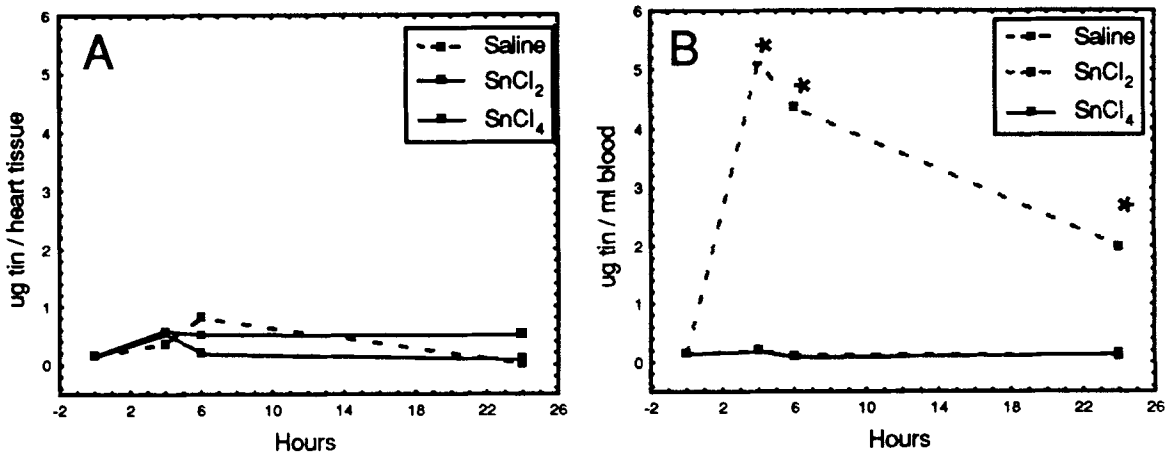


Fig. 2. Tin (μg) located per gram of heart tissue (**A**) or per ml blood (**B**) following treatment with saline, $SnCl_2$ (500 μmol/kg in saline) or $SnCl_4$ (500 μmol/kg in saline). Amount of tin determined by Atomic Absorption Spectroscopy as discussed in Materials and Methods. *$P < 0.05$ (as compared with the saline).

thus indicates that the metal was precipitated at the site of injection.

We next examined three different formulations of tin—citrate, phosphate, and tris—on induction of heme oxygenase activity in heart tissue and on the distribution of the metal. Tin citrate (both stannous and stannic) significantly induced heme oxygenase in heart tissue as compared with control rats (vehicle-citrate solution). As seen in Figure 3, the administration of stannous and stannic citrate resulted in a 3.7-fold and 1.8-fold increase, respectively, in heart heme oxygenase activity, after 16 h. This effect correlated with a several-fold induction of heme oxygenase-1 mRNA (data not shown). Figure 4 shows that 16 h after administration of stannic citrate there was 5.60 ± 1.05 μg tin/g heart tissue, while there was 1.32 ± 0.39 μg tin/g tissue following administration of stannous chloride.

The presence of citrate thus somehow facilitated the transport of the stannic ion from the site of administration and possibly facilitated the transport of both ions across cardiac cell membranes. This study indicates that both the stannous and stannic ions are capable of inducing heme oxygenase in the heart if administered in the form of citrate (as is also the case in the kidney), but although the Sn^{4+} citrate was present at a much higher level in the heart than the Sn^{2+} ion, the latter form of the metal was more potent at inducing HO-1 activity. Alternatively, the Sn^{4+} ion may be partially converted to the Sn^{2+} ion, although the data of Chiba and Kikuchi [1978] would appear to exclude this possibility.

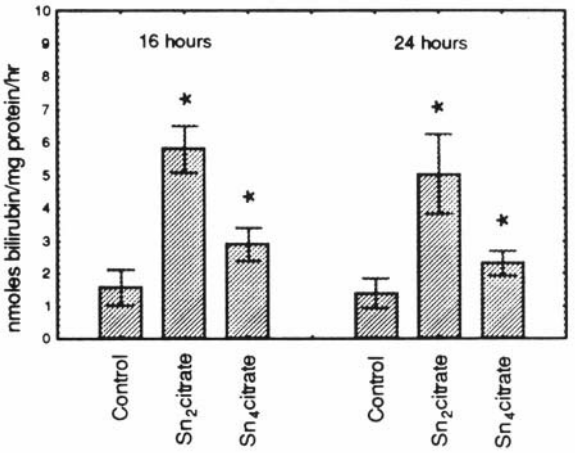


Fig. 3. Heme oxygenase activities in the heart ± standard deviation, 16 and 24 h after treatment with control (1,500 μmol sodium citrate/kg), Sn_2citrate (500 μmol $SnCl_2$/kg + 1,500 μmol sodium citrate/kg), Sn_4citrate (500 μmol $SnCl_4$/kg + 1,500 μmole sodium citrate/kg). Heme oxygenase activity was determined as discussed in Materials and Methods. *$P < 0.05$ (as compared with the control).

A valency effect on heme oxygenase has been noted previously. Sardana et al. [1981] showed that the arsenite ion (As^{3+}) was a more potent inducer of hepatic and renal heme oxygenase than was the arsenate (As^{5+}). Renal and hepatic heme oxygenase were also more responsive to the trivalent antimony ion (Sb^{3+}) than the pentavalent form (Sb^{5+}) [Drummond and Kappas, 1981]. In the case of the tin ion, Mitani et al. [1993] also reported that stannous chloride in-

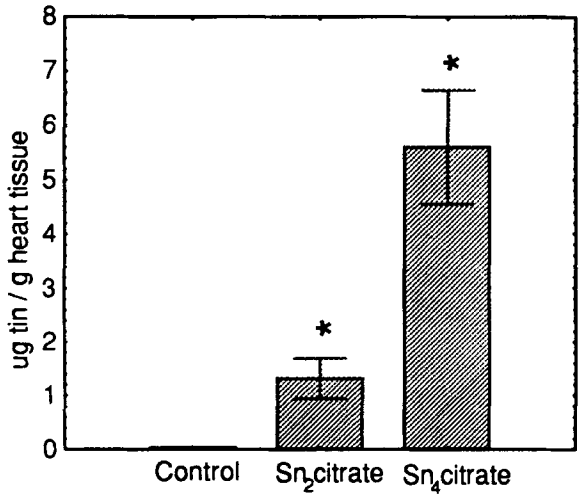


Fig. 4. Tin (μg) located per gram of heart tissue 16 h following treatment with control (1,500 μmol sodium citrate/kg), Sn_2citrate (500 μmol $SnCl_2$/kg + 1,500 μmol sodium citrate/kg), Sn_4citrate (500 μmol $SnCl_4$/kg + 1,500 μmol sodium citrate/kg). Amount of tin determined by Atomic Absorption Spectroscopy as discussed in Materials and Methods. *$P < 0.05$ (as compared with the saline).

duces heme oxygenase in hepatoma cells, whereas stannic chloride does not, and suggested that the stannous ion may place more oxidative stress on the cell than the stannic ion.

Our results suggest that the stannous ion may be more stressful to the cells since heme oxygenase is induced in response to increased oxidative stress in tissues. The relationship between heme oxygenase induction and oxidative stress is important in cardiac cells. The increase in free radicals upon reperfusion has been implicated in reperfusion injury in the heart [Kloner et al., 1989]. The induction of heme oxygenase during oxidative stress thus may be a defense mechanism, acting to restore the antioxidant/prooxidant ratio inside the cell [Stocker et al., 1987; Wu et al., 1991].

ACKNOWLEDGMENTS

The authors thank Stanly Huth, John Baker for their suggestions on various preparations, and Stephen Persell for his technical assistance. This work was supported in part by a grant from the NIH (HL54138-01) and by gifts from the Ogden Corporation, the Renfield Foundation, and the Kimmle Foundation.

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