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Journal

Toxicology and applied pharmacology, 112(1)

ISSN

0041-008X

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Publication Date

1992

DOI

10.1016/0041-008x(92)90292-z

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SHORT COMMUNICATION

Deferoxamine Inhibits Methyl Mercury-Induced Increases in Reactive Oxygen Species Formation in Rat Brain¹

Deferoxamine Inhibits Methyl Mercury-Induced Increases in Reactive Oxygen Species Formation in Rat Brain. LEBEL, C. P., ALI, S. F., AND BONDY, S. C. (1992). *Toxicol. Appl. Pharmacol.* 112, 161-165.

It has been suggested that methyl mercury may express its neurotoxicity by way of iron-mediated oxidative damage. Therefore, the effect of deferoxamine, a potent iron-chelator, on methyl mercury-induced increases in reactive oxygen species formation was studied in rat brain. The generation rate of reactive oxygen species was estimated in crude synaptosomal fractions using the probes 2',7'-dichlorofluorescein diacetate and dihydrorhodamine 123. The formation rate of the fluorescent oxidation products was used as the measure of reactive oxygen species generation. Seven days after a single injection of methyl mercury (5 mg/kg, ip), the formation rate of reactive oxygen species was significantly increased in the cerebellum. Pretreatment with deferoxamine (500 mg/kg, ip) completely prevented the methyl mercury-induced increase in cerebellar reactive oxygen species generation rates. The oxidative consequences of *in vitro* exposure to methyl mercury (20 μ M) were also inhibited by deferoxamine (100 μ M). The formation of the iron-saturated complex ferrioxamine was not affected by a 10-fold excess of methylmercuric chloride or mercuric chloride, suggesting that a deferoxamine-mercurial complex does not form. The findings in this study: (1) provide evidence that iron-catalyzed oxygen radical-producing reactions play a role in methyl mercury neurotoxicity, (2) demonstrate the potential of fluorescent probes as a measure of reactive oxygen species formation, and (3) provide support for iron-chelator therapy in protection against xenobiotic-induced oxidative damage. © 1992 Academic Press, Inc.

Reactive oxygen species such as superoxide anion, hydrogen peroxide, ferryl ion, and hydroxyl radical, if present in excess, are thought to be initiators of peroxidative cell damage (Freeman and Crapo, 1982; Halliwell and Gutteridge, 1984, 1986). The nervous system is exquisitely sensitive to peroxidative damage since it is rich in oxidizable substrates such as lipids and catecholamines (Halliwell and Gutteridge, 1985). With this in mind, there is increasing evidence to suggest that oxidative damage-inducing free radicals may play a role in the underlying biochemical mechanisms involved in the neurotoxicity of methylmercuric chloride (MeHg; Ganther, 1978, 1980). Studies have reported that MeHg

stimulates lipid peroxidation (Yonaha *et al.*, 1983) and the lipoperoxidative effects of MeHg are mitigated by the antioxidant α -tocopherol (vitamin E; Halliwell and Gutteridge, 1985; Kasuya, 1975; Bondy and McKee, 1990). Additionally, this laboratory has recently demonstrated that both *in vivo* and *in vitro* exposures to MeHg increased the formation rate of reactive oxygen species in mouse brain (LeBel *et al.*, 1990), effects that were observed only in the cerebellum, the brain region believed to be selectively vulnerable to MeHg (Syverson *et al.*, 1981). The latter findings were demonstrated with the aid of the probe 2',7'-dichlorofluorescein diacetate (DCFH-DA), a stable nonfluorescent molecule that readily crosses cell membranes and is hydrolyzed by intracellular esterases to nonfluorescent 2',7'-dichlorofluorescein (DCFH; Bass *et al.*, 1983). DCFH is then rapidly oxidized by reactive oxygen species to highly fluorescent 2',7'-dichlorofluorescein (DCF). Several reactive oxygen species appear to be responsible for the oxidation of DCFH (LeBel *et al.*, 1991).

It has become increasingly apparent that transition metals such as iron and copper play critical roles in toxicological and pathological phenomena that lead to oxidative cell damage (for review see Aust *et al.*, 1985). To prevent such phenomena, various chelating agents have been employed to minimize the participation of transition metals. One such agent is the iron chelator deferoxamine which has a high affinity for ferric iron (Keberle, 1964) and has been used clinically in cases of iron overload (Summers *et al.*, 1979; Mahoney *et al.*, 1989).

Of toxicological significance is the fact that deferoxamine has been used to protect against the effects of lindane (Videla *et al.*, 1989), paraquat (Kohen and Chevion, 1985), diquat (Smith, 1987), ethanol (Abu-Murad and Nordmann, 1983; Nordmann *et al.*, 1987), acetaminophen, and carbon tetrachloride (Younes and Siegers, 1985). The protection afforded by deferoxamine has been estimated using a variety of methodologies. Its ability to inhibit lipid peroxidation has been assayed with the thiobarbituric acid colorimetric procedure (Smith, 1987; Younes and Siegers, 1985).

The advantage of utilizing DCFH-DA is that it provides a direct measure of the formation rate of reactive oxygen species and, therefore, conclusions regarding the role of these species are not based on secondary mechanisms such as lipid peroxidation. The aim of the present study was to employ deferoxamine to investigate the role of iron-catalyzed oxygen

¹ This work was supported by NIH Grants ES 04071 and ES 07157.

radical reactions in rat brain, measured using fluorescent probes, following *in vivo* and *in vitro* exposure to MeHg.

MATERIALS AND METHODS

Materials. DCFH-DA, dihydrorhodamine 123 (DHR 123), and rhodamine 123 (R123) were obtained from Molecular Probes, Inc. (Eugene, OR), and DCF was purchased from Polysciences, Inc. (Warrington, PA). Deferoxamine mesylate was obtained from Sigma Chemical Co. (St. Louis, MO), and MeHg from ICN Pharmaceutical, K&K Labs (Plainview, NY).

Animals and treatment. Male CR 1 CD rats (National Center for Toxicological Research Breeding Colony, Jefferson, AR) weighing 215–235 g were employed in this study. Rats were housed two per cage with wood chip bedding and maintained on a 12-h light/dark cycle in a temperature-controlled ($20 \pm 1^\circ\text{C}$) room. Food (Purina Laboratory Chow, St. Louis, MO) and tap water were provided *ad libitum*. MeHg was dissolved in deionized-distilled water and administered in a single dose of 5.0 mg/kg intraperitoneally in a volume of 2 ml/kg. Deferoxamine was dissolved in deionized-distilled water and administered in a single intraperitoneal dose of 500 mg/kg (2 ml/kg dose volume) 1 hr before the administration of MeHg. All animals were euthanized seven days after treatment.

Preparation of P2 fractions. Rats were euthanized, the brains excised quickly on ice, and the cerebellum, cortex, and brainstem were dissected out. Each region was placed into a microcentrifuge tube, placed at -20°C for 24 hr, and stored at -70°C until crude synaptosomal (P2) preparation. Each region was weighed and homogenized in 10 vol of 0.32 M sucrose. The crude nuclear fraction was removed by centrifugation at 1800g for 10 min at 2°C ($r_{\text{av}} = 11.0$ cm). The resulting supernatant fraction was centrifuged at 31,500g for 10 min ($r_{\text{av}} = 11.0$ cm) to yield the P2 pellet. The P2 pellet was suspended in Hepes buffer to a concentration of 0.0185 g eq/ml. The composition of Hepes buffer was (mM): NaCl, 120; KCl, 2.5; NaH_2PO_4 , 1.2; MgCl_2 , 0.1; NaHCO_3 , 5.0; glucose, 6.0; CaCl_2 , 1.0 and; Hepes, 10.

Assay for reactive oxygen species. Quantification of the formation rate of oxygen reactive species was determined as previously described (LeBel *et al.*, 1990). Briefly, P2 fractions were diluted 1:10 with 40 mM Tris (pH 7.4) and loaded with $5 \mu\text{M}$ DCFH-DA or $10 \mu\text{M}$ DHR 123 (in methanol) for 15 min at 37°C . Following loading, the fluorescence was recorded prior to (initial) and after an additional 60 min (final) incubation. In studies that investigated the *in vitro* effects of MeHg (20 μM) and deferoxamine (10–100 μM), the agents were added to P2 fractions (isolated from rat cerebellum) after the initial fluorescence reading. Samples were then incubated for 60 min, followed by recording of the final fluorescence value. The formation of the fluorescent-oxidized derivatives of DCFH and DHR 123, namely DCF and R123, were monitored as follows: DCF, excitation wavelength, 488 nm (bandpass 5 nm), and emission wavelength, 525 nm (bandpass 20 nm); R123, excitation wavelength, 505 nm (bandpass 5 nm) and emission wavelength, 536 nm (bandpass 20 nm). The cuvette holder was thermostatically maintained at 37°C . Autofluorescence of P2 fractions was always less than 6%, and any methanol vehicle effect was recorded and subtracted before calculation of the formation of DCF and R123. DCF and R123 formation were quantified from separate standard curves in methanol (0.05–1.0 μM).

Assay for ferrioxamine formation. The chelation of deferoxamine and FeSO_4 was determined by the formation of ferrioxamine, adapted from the method of Hallaway *et al.* (1989). In a total volume of 2 ml, a 5-fold excess of FeSO_4 (final concentration 625 μM) was added to a solution of deferoxamine (final concentration 125 μM) in deionized-distilled water. To determine whether deferoxamine was able to chelate MeHg or Hg^{2+} , a 10-fold excess of either MeHg or HgCl_2 (final concentration 1.25 mM) was also employed. Each solution was allowed to incubate at room temperature (22°C) for 60 min, and the ferrioxamine complex formed was measured spectrophotometrically at 429 nm.

Statistical analyses. Differences between groups were assessed by either Student's *t* test or one-way analysis of variance followed by Fisher's least significant difference test. The acceptance level of significance was $p < 0.05$ using a two-tailed distribution. Five or six rats were used in the determination of each data point.

RESULTS AND DISCUSSION

The generation rates of cerebellar reactive oxygen species following *in vivo* exposure to MeHg and deferoxamine are presented in Fig. 1. Seven days following a single injection of MeHg (5 mg/kg) the formation rate of reactive oxygen species in P2 fractions isolated from rat cerebellum, as measured by DCF formation, was significantly increased (Fig. 1A). This finding is in agreement with our previous finding that MeHg stimulated the formation rate of reactive oxygen species in mouse cerebellum (LeBel *et al.*, 1990). The data also confirm reports that in both species of rodent, the cerebellum is the brain region that is selectively vulnerable to MeHg-induced morphological damage following *in vivo* exposure (Syverson *et al.*, 1981).

We have previously demonstrated that the chelating agent deferoxamine prevents oxidation of DCFH induced by a

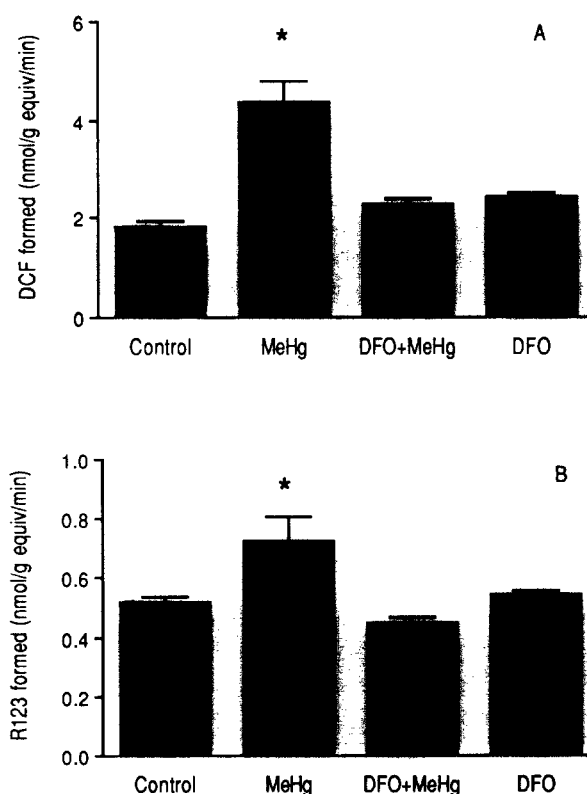


FIG. 1. The formation rate of cerebellar reactive oxygen species measured by DCF (A) or R123 (B) formation in P2 fractions isolated from animals 7 days after a single exposure to MeHg (5 mg/kg, ip). Values are the mean \pm SE, derived from six or seven animals/group. In some groups, deferoxamine (DFO, 500 mg/kg, ip) was given 1 hr prior to MeHg injection. *Significant difference from control value.

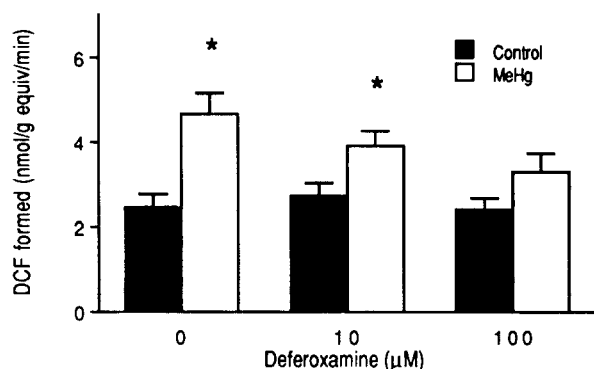


FIG. 2. Effects of deferoxamine treatment *in vitro* on the generation rate of cerebellar reactive oxygen species induced by MeHg in P2 fractions were isolated from untreated animals. Data were obtained from three independent experiments and are expressed as the mean \pm SE. *Significant difference from corresponding control value.

mixture of iron + ascorbate in rat cerebrocortical synaptosomes (LeBel and Bondy, 1990). This finding, taken together with the known protective effects of deferoxamine treatment in various toxicological phenomena (Younes and Siegers, 1985; Kohen and Chevion, 1985; Smith, 1987; Nordmann *et al.*, 1987; Videla *et al.* 1989), led us to investigate the potential amelioration of MeHg-induced changes in the central nervous system. The *in vivo* effect of MeHg was completely prevented by pretreatment with deferoxamine (500 mg/kg, 1 hr prior to MeHg injection) (Fig. 1A). Treatment with deferoxamine alone did not significantly alter the formation rate of reactive oxygen species compared to controls. MeHg, deferoxamine, or the combination did not significantly affect the generation rates of reactive oxygen species in either the cerebral cortex or the brainstem (data not shown).

The *in vitro* effects of MeHg and deferoxamine on the generation rates of reactive oxygen species were also studied. MeHg (20 μ M) significantly increased reactive oxygen species formation in cerebellar P2 fraction isolated from control rats (Fig. 2). Addition of deferoxamine *in vitro* (100 μ M) inhibited MeHg-induced increases in cerebellar reactive oxygen species formation, as was the case following *in vivo* exposure to MeHg, while 10 μ M deferoxamine attenuated MeHg's effect. Higher concentrations of deferoxamine (1 mM) completely blocked MeHg stimulation of reactive oxygen species generation; however, control formation rates were also inhibited (data not shown).

Despite the fact that deferoxamine is known to be selective for iron, a possible mechanism by which deferoxamine inhibited MeHg-induced increases in reactive oxygen species generation may have been via chelation of the Hg moiety of MeHg. Therefore, the potential of deferoxamine to chelate MeHg or Hg^{2+} was investigated by looking at the formation of the iron-saturated deferoxamine complex, ferrioxamine.

Even in the presence of a 10-fold excess of either MeHg or HgCl_2 , the formation of ferrioxamine was not inhibited (Table 1). No complex-generating absorbance was noted with deferoxamine and MeHg or HgCl_2 in the absence of iron. Although the experiments performed are not unequivocal regarding the ability of deferoxamine to bind mercury, it is unlikely that there are significant amounts of inorganic mercury salts present 1 hr after the administration of MeHg (Racz and Vandewater, 1982). Treatment with deferoxamine alone did not affect the basal rate of formation of reactive oxygen species in rat cerebellum (Fig. 1). In fact, animals pretreated with deferoxamine and subsequently exposed to MeHg appeared to be in better general health (healthier coats, less perinasal staining and chromodacryorrhea) than the group receiving no such treatment before MeHg injection. While deferoxamine has shown some acute toxicity in clinical studies (Whitten *et al.*, 1965, 1966; Westlin, 1966), its therapeutic potential demonstrated in the present study and against other xenobiotic-induced toxicities appears promising.

Since the formation of DCFH from DCFH-DA requires the action of intracellular esterases, an alternative probe was used to determine whether MeHg treatment affected intracellular esterase activity. The probe DHR 123 is oxidized by reactive oxygen species to its fluorescent product R123, a process that does not require esterases. *In vivo* exposure to MeHg significantly increased reactive oxygen species formation, as measured by R123 fluorescence, in rat cerebellum, which was also blocked by pretreatment with deferoxamine (Fig. 1B). Again, treatment with deferoxamine alone was without effect. Therefore, the increased rate of formation of DCF following exposure to MeHg did not appear to be related to an effect on esterase activity since the formation rate of R123, which does not require esterases, was also stimulated by MeHg. R123, because of its positive charge, has been used as a measure of mitochondrial membrane potential in

TABLE 1
Effect of Methylmercuric Chloride on the Formation of Ferrioxamine

Incubation mixture	Absorbance at 429 nm ^a
125 μ M deferoxamine + 625 μ M FeSO_4	0.347 \pm 0.005
125 μ M deferoxamine + 625 μ M FeSO_4 + 1.25 mM MeHg	0.357 \pm 0.004
125 μ M deferoxamine + 1.25 mM MeHg	≤ 0.001
125 μ M deferoxamine + 625 μ M FeSO_4 + 1.25 mM HgCl_2	0.345 \pm 0.007
125 μ M deferoxamine + 1.25 mM HgCl_2	≤ 0.001

^a Each value represents the mean \pm SE from three independent measurements.

nerve terminals (Yoshikami and Okun, 1984; Magrassi *et al.*, 1987) and in liver (O'Connor *et al.*, 1988). Preliminary experiments performed in our laboratory indicate that DHR 123 is oxidized to fluorescent R123 in a similar fashion as DCFH (C. P. LeBel and C. S. Bondy, unpublished observations), suggesting that it may also be used as a measure of reactive oxygen species. The localization of R123 in mitochondria (Yoshikami and Okun, 1984; Magrassi *et al.*, 1987), together with findings of the present study and the fact that brain mitochondria are a rich source of reactive oxygen species (Cino and Del Maestro, 1989), supports the theory that mitochondria may be a specific cellular locus for the critical action of MeHg (Cheung and Verity, 1981).

The results obtained with the fluorescent markers used here suggest a role for reactive oxygen species in the mechanism of MeHg neurotoxicity. DCF and R123 formation also appear dependent on the presence of both an iron-peroxide complex (ferryl ion) and other reactive oxygen species (LeBel *et al.*, 1991), which further implicates iron-catalyzed oxygen radical reactions in MeHg toxicity. Furthermore, the use of DCF as a marker for MeHg-induced oxidative cell damage appears to be a most sensitive measure when compared to standard lipoperoxidative techniques. MeHg stimulated the formation of DCF at least two-fold (Figs. 1 and 3), while previous studies in synaptosomes using thiobarbituric acid-reactive products (TBARs) only showed modest increases in TBARs in the presence of 25 μ M MeHg (Bondy and McKee, 1990).

Most theories regarding the toxicity of MeHg work on the premise that the intact molecule interacts with protein sulfhydryl groups to produce its toxicity (Ganther, 1978, 1980). An alternative hypothesis is that MeHg undergoes cleavage, leading to the formation of Hg^{2+} and a methyl radical (Ganther, 1980). It is not likely that the observed increases in DCF formation are due to Hg^{2+} since it has been shown that less than 4% of the mercury burden in the brain existed as inorganic mercury on the tenth day following intravenous injection of MeHg (Norseth and Clarkson, 1970; Raczy and Vanderwater, 1982). Regarding the possibility that methyl radical might play a role, its formation is reported to be induced by ascorbate and copper and is thought to be catalyzed by a copper-mediated free radical mechanism (Gage, 1975). The methyl radical, or other radical species, may therefore be linked to the increased oxidation of DCFH following exposure to MeHg.

As mentioned previously, there is an increasing body of information to suggest that one of the mechanisms in the neurotoxicity of MeHg involves iron-catalyzed oxygen radical-induced cell damage. The present study not only supports this theory, but specifically suggests that an iron-peroxide intermediate (ferryl ion) and other reactive oxygen species may play a major role in MeHg-induced cell death. MeHg not only appears to increase the formation of ferryl

ion, but several other studies have implicated this reactive oxygen complex as the most probable damage-inducing species (Arai *et al.*, 1987; Yamamoto and Kawanishi, 1989). The postulate has been raised that reactive oxygen species may act as chemical messengers (Saran and Bors, 1989) further supporting the concept that these species may play a critical role in various mechanisms of neurotoxicity (Bondy and LeBel, 1991; LeBel and Bondy, 1991). The fact that parallel *in vivo* and *in vitro* effects are observed in studies that show oxygen radical generation enhancement by MeHg and for several other known neurotoxicants (LeBel and Bondy, 1991) demonstrates the usefulness of these fluorescent dyes as sensitive markers for neurotoxicity.

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Received October 3, 1991; accepted October 6, 1991

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