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**Title:**
Organometal-induced increases in oxygen reactive species: The potential of 2′,7′-dichlorofluorescin diacetate as an index of neurotoxic damage

**Journal Issue:**
Toxicology and Applied Pharmacology, 104(1)

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**Publication Date:**
06-01-1990

**Series:**
UC Irvine Previously Published Works

**Permalink:**
http://escholarship.org/uc/item/319481c0

**DOI:**
https://doi.org/10.1016/0041-008X(90)90278-3

**Local Identifier(s):**
UCPMS ID: 345610

**Abstract:**
The effects of the neurotoxic metals methylmercury (MeHg) and trimethyltin (TMT) on oxygen reactive species formation within a crude synaptosomal fraction (P2), using the probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA), and intracellular calcium ([Ca 2+ ] i ), with the fluorescent indicator fluo-3, have been investigated. Two and seven days after a single injection of MeHg (1 mg/kg) the formation rate of cerebellar oxygen reactive species was significantly increased. Hippocampal and frontocortical oxygen reactive species were elevated 2 days after TMT injection (3 mg/kg). In vitro exposure to MeHg (10-20 μm) increased the formation rate of oxygen reactive...
species, while TMT (5-40 μm) was without effect. Levels of [Ca 2+ ] i were unaltered in P2 fractions from cerebellum and hippocampus of animals treated with either organometal. The data demonstrate that oxygen reactive species are elevated in brain regions, cerebellum (MeHg) and hippocampus (TMT), believed to be selectively vulnerable to these toxic agents. Findings suggest that oxidative damage may be a mechanism underlying the toxicity of both organometals. The use of DCFH-DA may have potential in the nervous system as an indicator of neurotoxic damage. © 1990.

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Organometal-Induced Increases in Oxygen Reactive Species: The Potential of 2',7'-Dichlorofluorescin Diacetate as an Index of Neurotoxic Damage

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Received September 29, 1989; accepted January 30, 1990

Organometal-Induced Increases in Oxygen Reactive Species: The Potential of 2',7'-Dichlorofluorescin Diacetate as an Index of Neurotoxic Damage. LEBEL, C. P., ALI, S. F., MCKEE, M., AND BONDY, S. C. (1990). Toxicol. Appl. Pharmacol. 104, 17-24. The effects of the neurotoxic metals methylmercury (MeHg) and trimethyltin (TMT) on oxygen reactive species formation within a crude synaptosomal fraction (P2), using the probe 2',7'-dichlorofluorescin diacetate (DCFH-DA), and intracellular calcium ([Ca2+]i), with the fluorescent indicator fluo-3, have been investigated. Two and seven days after a single injection of MeHg (1 mg/kg) the formation rate of cerebellar oxygen reactive species was significantly increased. Hippocampal and frontocortical oxygen reactive species were elevated 2 days after TMT injection (3 mg/kg). In vitro exposure to MeHg (10-20 µM) increased the formation rate of oxygen reactive species, while TMT (5-40 µM) was without effect. Levels of [Ca2+]i were unaltered in P2 fractions from cerebellum and hippocampus of animals treated with either organometal. The data demonstrate that oxygen reactive species are elevated in brain regions, cerebellum (MeHg) and hippocampus (TMT), believed to be selectively vulnerable to these toxic agents. Findings suggest that oxidative damage may be a mechanism underlying the toxicity of both organometals. The use of DCFH-DA may have potential in the nervous system as an indicator of neurotoxic damage. © 1990 Academic Press, Inc.

The organometals methylmercury (MeHg) and trimethyltin (TMT) are extremely toxic to the central nervous system, but the nature of the underlying biochemical mechanisms that lead to impaired cell function and neuronal death remains unclear. Investigations into the neurotoxicity of MeHg have reported inhibition of protein synthesis (Cheung and Verity, 1985), uncoupling of oxidative phosphorylation (Cheung and Verity, 1981), decreased intracellular ATP (Sarafian et al., 1984), and increased intrasynaptosomal Ca2+ ([Ca2+]i) (Komulainen and Bondy, 1987; Bondy and Mckee, 1990). Morphological changes following exposure to MeHg are especially pronounced in the cerebellum where granule cells are selectively damaged (Syverson et al., 1981). In addition, more widespread pathological changes are apparent (Komulainen, 1988).

TMT toxicity has been proposed to be related to the stimulation of an exchange of Cl− for OH− ions across inner mitochondrial membranes (Aldridge et al., 1977). TMT also inhibits mitochondrial ATP synthesis (Aldridge, 1976; Aldridge et al., 1977), increases [Ca2+]i (Komulainen and Bondy, 1987), and reduces γ-aminobutyric acid concentrations (Mailman et al., 1983) and neurotransmitter

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uptake (Doctor et al., 1982). A special vulnerability of the hippocampal formation to TMT is morphologically apparent (Chang et al., 1982a,b) and this has been correlated with distinctive behavioral changes (Walsh and DeHaven, 1988).

It has been suggested that MeHg may express its toxicity by way of oxidative damage. MeHg stimulates hepatic and renal lipid peroxidation (Yonaha et al., 1983), and its effects on neural development are mitigated by the antioxidant α-tocopherol (vitamin E) in cell culture (Kasuya, 1975). Furthermore, MeHg itself may form oxidative damage-inducing free radicals (Ganther, 1978, 1980). While no specific oxidative component has been identified in TMT-induced damage, triorganotin compounds appear to have severe membrane-damaging properties (Byington et al., 1974).

Oxygen reactive species, such as superoxide anion, hydrogen peroxide, and hydroxyl radicals, are believed to be initiators of oxidantive cell damage (Halliwell and Gutteridge, 1984, 1986). Recently, a fluorescent technique using 2',7'-dichlorofluorescin diacetate (DCFH-DA) has been used to quantitate cerebral oxygen reactive species (LeBel et al., 1989; LeBel and Bondy, 1990). DCFH-DA is a stable, nonfluorescent molecule that readily crosses cell membranes, and is hydrolyzed by intracellular esterases to nonfluorescent 2',7'-dichlorofluorescin (DCFH) (Bass et al., 1983). DCFH is then rapidly oxidized in the presence of oxygen reactive species to highly fluorescent 2',7'-dichlorofluorescein (DCF). The present study employed DCFH-DA to test two separate hypotheses: (1) Do oxygen reactive species play a role in the neurotoxic mechanisms following in vivo and in vitro exposure to MeHg and TMT? (2) Is DCFH-DA a potential marker for neurotoxicity, and how does it compare to the use of levels of free [Ca2+]i, another potential marker for neurotoxic damage (Komulainen and Bondy, 1987, 1988), since [Ca2+]i and oxidative stress appear to be related by reciprocal potentiation (Thor et al., 1984; Richter and Frei, 1988; Nicotera et al., 1988)?

METHODS

Animals and treatment. Male C57Bl/6N mice (National Center for Toxicological Research Breeding Colony, Jefferson, AR), 8–10 weeks old and weighing 20–25 g, were employed in this study. Mice were housed four per cage with wood-chip bedding and maintained on a 12-hr light/dark cycle in a temperature-controlled (20 ± 1°C) room. Food (Purina Laboratory Chow, St. Louis, MO) and tap water were provided ad libitum.

MeHg and TMT (ICN Pharmaceutical, K&K Labs, Plainview, NY) were dissolved in deionized–distilled water and administered in single doses of 1.0 mg/kg (MeHg) and 3.0 mg/kg (TMT) intraperitoneally in a volume of 10 ml/kg. MeHg-treated animals were euthanized 2 and 7 days following exposure, and TMT-treated animals were euthanized 1 and 2 days postdose. The rationale for the doses and time-course used was essentially threefold: (1) to maintain the overall health of the animals, (2) to employ doses that would not necessarily lead to overt neuropathological effects, and (3) to study early neurochemical events. At the doses used, mice given MeHg had no remarkable central nervous system effects, while approximately 50% of the animals given TMT experienced tremor (Ali et al., 1986).

Chemicals. DCFH-DA and fluo-3/AM were obtained from Molecular Probes, Inc. (Eugene, OR), and DCF was purchased from Polysciences, Inc. (Warrington, PA).

Preparation of P2 fractions. Mice were decapitated, the brains were excised quickly on ice, and the hippocampus, frontal cortex, cerebellum, 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 P2 preparation. Each region was weighed and homogenized in 10 vol of 0.32 M sucrose. The crude mitochondrial fraction (P1) was removed by centrifugation at 1800g for 10 min. The resulting supernatant fraction (S1) was centrifuged at 31,500g for 10 min to yield the crude mitochondrial pellet (P2). 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; NaH2PO4, 1.2; MgCl2, 0.1; NaHCO3, 5.0; glucose, 6.0; CaCl2, 1.0; and Hepes, 10.

Assay for oxygen reactive species. P2 fractions were diluted 1:10 with 40 mM Tris (pH 7.4) and loaded with 5 μM DCFH-DA (in methanol) for 15 min at 37°C, during which time esteratic activity results in the formation the nonfluorescent compound DCFH (Bass et al., 1983). 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 the
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organometals, MeHg and TMT were added to P2 fractions (isolated from whole brain) in various concentrations after the initial fluorescence reading. Samples were incubated for 60 min, followed by recording of the final fluorescence value. The formation of the fluorescent oxidized derivative of DCFH, namely DCF, was monitored at excitation wavelength 488 nm (bandpass 5 nm) and emission wavelength 525 nm (bandpass 20 nm). The cuvette holder was thermostatically maintained at 37°C. Autofluorescence of P2 fractions, always less than 6%, and any methanol vehicle effect were recorded and subtracted before calculation of the formation of DCF. DCF formation was quantified from a standard curve in methanol (0.05–1.0 µM).

Assay for \([\text{Ca}^{2+}]\). The measurement of \([\text{Ca}^{2+}]\) was performed using the acetoxyethyl ester of the indicator dye fluo-3/AM (Kao et al., 1989). Aliquots (0.5 ml) of P2 suspension were incubated with 10 µM fluo-3/AM added in dimethyl sulfoxide (DMSO) (final concentration of DMSO, 0.5%) at 37°C for 30 min. The DMSO also contained a nonionic surfactant, Pleuronic F-127 (20%, v/v), in order to aid solubilization of the fluo-3 ester. Hepes buffer (9 ml) was then added and P2 fractions were sedimented (12500g, 8 min). The resulting pellets were resuspended in 1.5 ml of Hepes buffer and kept on ice. For fluorescence measurements, 0.5-ml aliquots of the suspensions were diluted to 2 ml in warm (37°C) Hepes buffer. This buffer did not contain NaHCO3 or Na2HPO4, in order to prevent precipitation of high levels of Ca2+ used subsequently. The emitted fluorescence of the hydrolyzed fluo-3 ester in the sample was measured in a thermostated (37°C) cuvette using a spectrofluorometer at the excitation wavelength of 506 nm (bandpass 3 nm) and at 526 nm for emission (bandpass 10 nm). All incubations were at 37°C, and mixing was carried out with a magnetic stirrer 30–60 sec before fluorescence was read. Autofluorescence of P2, always less than 15%, was recorded and subtracted before calculation of \([\text{Ca}^{2+}]\). For calibration of fluo-3-Ca2+ signal, \(F,F_{\text{min}}\) (fluorescence in the absence of Ca2+), and \(F_{\text{max}}\) (fluorescence from Ca2+-saturated fluo-3) were determined for each batch of fluo-3-loaded P2. In order to determine \(F_{\text{min}}\), P2 membranes were lysed with 0.1% sodium dodecyl sulfate, and Ca2+ and Mn2+ were chelated with 5 mM alkaline ethylene glycol tetraacetic acid (EGTA). \(F_{\text{max}}\) was determined by the addition of 9 mM CaCl2. \([\text{Ca}^{2+}]\) was calculated using the formula \([\text{Ca}^{2+}] = K_d(F - F_{\text{min}})/(F_{\text{max}} - F)\), where \(K_d\) (400 nM) is the dissociation constant of fluo-3-Ca2+ complex (Kao et al., 1989).

Statistical analysis. Analysis of variance (ANOVA) was used to determine statistical difference between treatments, followed by a Student t test with \(p < 0.05\) taken as significant.

RESULTS

The formation of cerebral P2 oxygen reactive species was studied following acute in vivo exposure to low doses of MeHg or TMT. The crude mitochondrial preparation, a potent source of oxygen reactive species, was used since it was recently shown that superoxide dismutase activity is higher in synaptic than in nonsynaptic mitochondria (Vanella et al., 1989). MeHg significantly increased the formation rate of oxygen reactive species (as judged by increased DCF fluorescence) in the cerebellum 2 and 7 days after treatment (Fig.

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FIG. 1. The time-course effects of in vivo MeHg administration on the rate of formation of oxygen reactive species in various brain regions. Data are expressed as means ± SE derived from eight mice. Zero time represents control levels. Asterisks denote statistical difference from controls following Student's t test at \(p < 0.05\).
FIG. 2. The time-course effects of in vivo TMT administration on the rate of formation of oxygen reactive species in various brain regions. Data are expressed as means ± SE derived from eight mice. Zero time represents control levels. Asterisks denote statistical difference from controls following Student’s t test at $p < 0.05$.

1). DCF formation was not affected by MeHg in the hippocampus, frontal cortex, or brainstem (Fig. 1). TMT (2 days) significantly increased the formation rate of oxygen reactive species in the hippocampus and frontal cortex, with no observable effect in the cerebellum or brainstem (Fig. 2). In control animals, frontal cortex and brainstem had consistently higher basal rates of formation of oxygen reactive species than hippocampus and cerebellum (Figs. 1 and 2).

To determine whether these neurotoxic organometals directly increase the formation of oxygen reactive species, MeHg and TMT, at various concentrations, were incubated with whole-brain P2 fractions in vitro. Increases in oxygen reactive species occurred in the presence of low concentrations of MeHg ($10 \mu$M), with maximal effects observed with $20 \mu$M MeHg (Fig. 3). TMT did not alter the formation of oxygen reactive species at any of the concentrations employed (Fig. 3). Whole-brain P2 fractions were used in this study since preliminary experiments done in our laboratory have not detected any regional differences following in vitro exposures to these agents (data not shown).

It was previously demonstrated that synapticosomal $[\text{Ca}^{2+}]_i$ was increased following in vitro incubations in the presence of MeHg and TMT (Komulainen and Bondy, 1987; Bondy and McKee, 1990). Since numerous reports had shown selective vulnerability of hippocampus and cerebellum to TMT and MeHg, respectively, $[\text{Ca}^{2+}]_i$ was measured after in vivo exposure in these regions. $[\text{Ca}^{2+}]_i$ levels in P2 fractions derived from hippocampus or cerebellum were not affected by either MeHg or TMT (Fig. 4).

**DISCUSSION**

The role of oxygen reactive species formation as an early posttraumatic event following spinal cord injury has been described (Demopoulos et al., 1982; Milvy et al., 1973). The present study suggests that a mechanism for the neurotoxicity of MeHg may involve oxygen reactive species-induced damage. Two and seven days following in vivo administration of MeHg, significant increases in the formation rate of cerebellar oxygen reactive species were observed (Fig. 1). The fact that only the cerebellum was affected by MeHg is consistent with the findings on the regional selectivity of MeHg-induced morphological damage (Syverson et al., 1981). It may be that cerebellar cells are unable to withstand MeHg-induced increases in oxygen reactive
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FIG. 3. The formation rates of oxygen reactive species in cerebral P2 fractions following in vitro exposure to MeHg and TMT. Data were obtained from two independent experiments and are expressed as the mean with differences less than 7% between assays. Zero concentration represents control levels.

species formation. The cerebellum is reported to have lower endogenous vitamin E levels (Vatassery et al., 1984; Meydani et al., 1988; LeBel et al., 1989) and enhanced levels of peroxidative damage following vitamin E deficiency (Meydani et al., 1988). Vitamin E is reported to play a critical role in modifying MeHg-induced toxicity in cerebellar tissue cultures (Kasuya, 1975). MeHg, in vitro, also stimulated the rate of formation of oxygen reactive species (Fig. 3) to an extent similar to that observed in the intact animal (Fig. 1).

TMT also increased the formation rate of oxygen reactive species (Fig. 2). These findings are consistent with the vulnerability of the hippocampal formation to TMT (Chang et al., 1982a,b). The frontal cortex also showed increases in DCF formation (Fig. 2). However, in vitro incubations in the presence of TMT did not increase oxygen reactive species (Fig. 3). The increase in DCF fluorescence following in vivo exposure to TMT (Fig. 2) and MeHg (Fig. 1) may be related to phagocytic absorption of damaged neurons. Neuronophagia has been documented following TMT exposure (Bouldin et al., 1981; Reuhl et al., 1985). Although the phenomenon of increased oxygen reactive species generation has not been documented in the injured brain during neuronophagia, it is known that during phagocytosis oxidative bursts occur, leading to the release of oxygen reactive species (Rossi et al., 1982). DCF formation has been used to quantitate the oxidative burst process in polymorphonuclear leukocytes (Bass et al., 1983; Szejda et al., 1984).

[Ca\(^{2+}\)]\(_{\text{i}}\) was previously shown to be increased following in vitro exposure to MeHg (Komulainen and Bondy, 1987; Bondy and McKee, 1990). One component of the present study was to determine if in vivo exposure to MeHg and TMT altered [Ca\(^{2+}\)]\(_{\text{i}}\). Using the fluorescent indicator dye fluo-3, neither organometal altered [Ca\(^{2+}\)]\(_{\text{i}}\). It therefore appears that [Ca\(^{2+}\)]\(_{\text{i}}\) is not always sensitive to in vivo manipulations. Recently, it was reported that cyanide, both in vivo and in vitro, increased neuronal Ca\(^{2+}\) (Johnson et al., 1986, 1987). However, the in vivo studies measured only total brain Ca\(^{2+}\), and not [Ca\(^{2+}\)]\(_{\text{i}}\). Therefore, while [Ca\(^{2+}\)]\(_{\text{i}}\) may have value as an in vitro marker for neurotoxic damage (Komulainen and Bondy, 1987, 1988; Bondy and McKee, 1990), it appears to have limitations in vivo.

A potential application of DCFH-DA might be as a marker for early neurological damage. This probe has been used to measure oxygen reactive species in neuronal cell cultures (Saez et al., 1987; Murphy et al., 1989), and was recently characterized in synaptosomes (LeBel and Bondy, 1990). The present study has demonstrated the utility of DCFH-DA in an in vivo situation. The data suggest that these organometals induce increases in the rates of formation of oxygen reactive species in brain regions known to be specifically vulnerable to each neurotoxicant. The extent to which this is due to parallel underlying mechanisms is unclear. MeHg, but not TMT, was capable of inducing increased DCFH oxidation in an isolated system, as well as in vivo. In the case of MeHg, oxygen reactive species production may be directly induced, a theory in agreement with a study that showed
FIG. 4. Cytosolic concentration of ionic calcium \( [\text{Ca}^{2+}]_i \), in cerebellar and hippocampal P2 fractions isolated from mice treated with (a) MeHg or (b) TMT. Data are expressed as means ± SE derived from eight mice. No statistical differences were observed.

MeHg-induced lipoperoxidation in cerebellar granule cells (Verity and Sarafian, 1989). TMT may cause oxidative stress secondarily by way of compromise of systemic or cellular integrity. In support of this concept is the report that acute exposure to TMT induces hyperammonemia (Wilson et al., 1986). Therefore, the systemic insult observed following in vivo administration of TMT could lead to increased oxygen reactive species generation, an event that may not occur following in vitro exposure.

An emerging broader issue is the possibility that the metabolic disruption caused by diverse toxic agents, acting on various loci, may in part be expressed by abnormal levels of production of oxygen reactive species. Support for this concept is shown by a recent description of increases in oxygen reactive species formation (measured using DCFH-DA) following dietary vitamin E deficiency (LeBel et al., 1989). Therefore, within the nervous system this probe may have potential as a useful indicator of neurotoxic damage.

ACKNOWLEDGMENTS

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

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