Manganese Protects against Heart Mitochondrial Lipid Peroxidation in Rats Fed High Levels of Polyunsaturated Fatty Acids1,2,3

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ABSTRACT We demonstrated previously that dietary manganese (Mn) deficiency depressed Mn concentrations in most tissues and consistently depressed Mn superoxide dismutase (MnSOD) levels in heart. To examine the functional consequences of these effects, we fed weanling male Sprague-Dawley rats (n = 12/diet) diets containing 20% (wt/wt) corn oil or 19% menhaden oil + 1% corn oil by weight and 0.75 or 82 mg Mn/kg diet for 2 mo (the fish oil mixture was supplemented with +-(mixed)-α-tocopherol to the level in corn oil). Heart and liver Mn concentrations in the Mn-deficient rats were 56% of those in Mn-adequate rats (P < 0.0001), confirming Mn deficiency. The Mn-deficient rats had more conjugated dienes in heart mitochondria than Mn-adequate rats (P < 0.001); rats fed fish oil had more conjugated dienes than those fed corn oil (P < 0.001). The MnSOD activity was inversely correlated with conjugated dienes (r = −0.71, P < 0.005), and Mn-deficient rats had 37% less MnSOD activity in the heart than did Mn-adequate rats (P < 0.0001). The dietary treatments did not affect heart microsomal conjugated diene formation, possibly because of compensation by copper-zinc (CuZn) SOD activity; CuZnSOD activities were 35% greater in the hearts of Mn-deficient animals (P < 0.01). Liver was less sensitive to Mn deficiency than was the heart as judged by MnSOD activity and conjugated diene formation. This work is the first to demonstrate that dietary Mn protects against in vivo oxidation of heart mitochondrial membranes. J. Nutr. 126: 27–33, 1996.

INDEXING KEY WORDS:

• manganese  • superoxide dismutase  
• lipid peroxidation  • dietary fat  • rats

Activity of the mitochondrial antioxidant enzyme manganese superoxide dismutase (MnSOD, EC 1.15.1.1)5 is related to nutritional status in regard to manganese. Numerous investigators have noted that manganese-deficient rats had depressed tissue manganese concentrations, depressed MnSOD activity in heart, and sometimes depressed MnSOD activity in liver (Davis et al. 1990 and 1992, deRosa et al. 1980, Malecki et al. 1994, Zidenberg-Cherr et al. 1983). Young women consuming on average 1.7 mg manganese/d responded to 15 mg manganese/d supplementation with increased lymphocyte MnSOD activity (Davis and Greger 1992).

The functional effects of manganese deficiency with decreased MnSOD activity are less clear. Ultrastructural abnormalities but no changes in P/O ratios or oxygen uptake were noted in liver mitochondria of manganese-deficient mice and rats (Hurley et al. 1970, Zidenberg-Cherr et al. 1985), deRosa et al. (1980) and Paynter (1980) both showed that manganese protected tissues from oxidation (as measured by thiobarbituric acid–reactive substances [TBARS] when the tissues were incubated in vitro with oxidation initiators. However, such systems measure the inherent peroxidizability of the samples, not necessarily damage that would occur under physiological conditions (Recknagel et al. 1991). We hypothesized that manganese deficiency and the resultant decrease in MnSOD activity would make heart mitochondria more sensitive to in vivo oxidative damage.

Dietary fat influences the fatty acid profile of organelle membranes (Kuratko et al. 1994). Animals with highly polyunsaturated membranes are susceptible to

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4 To whom correspondence should be addressed.
5 Abbreviations used: CuZnSOD, copper-zinc superoxide dismutase [EC 1.15.1.1]; MnSOD, manganese superoxide dismutase; TBARS, thiobarbituric acid–reactive substances.
lipid peroxidation (Kuratko et al. 1994, L'Abbé et al. 1991). Thus, we hypothesized that rats fed highly polyunsaturated fatty acids would be more susceptible to the effects of manganese deficiency on in vivo lipid peroxidation.

MATERIALS AND METHODS

The study had a 2 × 2 (fat type × manganese level) factorial design. The four dietary treatments were menhaden oil and manganese-deficient, menhaden oil and manganese-adequate, corn oil and manganese-deficient, and corn oil and manganese-adequate. The diets contained 20% fat (wt/wt) so that effects due to the type of fat would be accentuated. The diets were formulated to provide the same amounts of protein, fiber, vitamins and minerals per 100 kJ consumed as the AIN-76 diet (AIN 1977).

The diets contained the following (g/100 g diet): lactalbumin [23.5]; sucrose [34.4; Kohl’s, Madison, WI]; cornstarch [10.33]; cellulose [5.88]; DL-methionine [0.35]; choline bitartrate [0.24]; AIN-76 vitamin mix [1.18]; AIN-76 mineral mix, with or without manganese [4.12]; and fat either as corn oil [20.0; Kohl’s] or as alkali-refined, bleached and pressed menhaden oil [19.0]; Zapata Protein, Reedville, VA) plus corn oil [1.0]. Diet ingredients other than those indicated were obtained from Teklad [Madison, WI]. By analysis, the manganese-deficient diets contained 0.75 ± 0.08 μg manganese/g diet, and the manganese-adequate diets contained 82 ± 7 μg manganese/g diet.

The menhaden oil diets were supplemented with +-(mixed)-α-tocopherol (Sigma Chemical, St. Louis, MO) to equal the concentration of +-(mixed)-α-tocopherol in corn oil (Scott and Latshaw 1991). Diets were prepared weekly. Dry ingredient mixes, oils and complete diets were stored at −20°C to inhibit oxidation.

Animals. Twelve male weaning Sprague-Dawley rats, weighing 49 ± 1 g initially [Harlan Sprague-Dawley, Madison, WI], were fed each diet for 8 wk. This number of animals was necessary to provide a sufficient quantity of heart tissue for the subcellular analyses. The rats were housed individually in stainless steel wire-bottomed cages and were allowed free access to triple distilled water. The protocol for this work and the facilities were approved by an institutional animal care and use committee.

The dietary treatments had no effect on body weights or food intakes of rats. Overall, the animals weighed 333 ± 5 g after 8 wk of the dietary treatments and consumed 14.1 ± 0.2 g of food daily.

Sample collection. Rats were deprived of food for 24 h before being anesthetized with CO2 and exsanguinated by cardiac puncture. Tissues to be analyzed for minerals [four hearts per treatment, tibias, liver right lobes, kidneys, spleens, pancreata, and 1-g samples of muscle] were cleaned of adhering material, weighed and frozen in new test tubes (Starstedt, Arlington Heights, IL).

Subcellular fractionation. Fresh hearts and portions of livers were homogenized in 10 volumes of buffer [0.25 mol/L sucrose, 10 mmol/L HEPES, 1 mmol/L EDTA, 1 mmol/L MgCl2] with a Tissumizer (Tekmar, Cincinnati, OH) and centrifuged at 1000 × g for 20 min. To obtain sufficient quantities of heart subcellular fractions, pooled samples were analyzed (two hearts/pool, giving four pooled samples per treatment). Aliquots of supernatant were frozen at −85°C for later enzyme analyses.

The remaining fresh supernatant was centrifuged at 20,000 × g for 10 min, giving a crude mitochondrial pellet. The pellet was resuspended in 2 mL of PBS. Lipids from 1 mL of the resulting suspension were extracted for conjugated diene determination [Bacon et al. 1983]. The remaining mitochondrial suspension was frozen for MnSOD activity measurements. The remaining supernatant was centrifuged at 100,000 × g for 30 min, giving a crude microsomal pellet from which lipids were extracted and analyzed for conjugated dienes.

Analyses. Manganese superoxide dismutase activities in mitochondria were measured by inhibition of pyrogallol autoxidation in the presence of cyanide [Marklund and Marklund 1974]. Copper-zinc superoxide dismutase [CuZnSOD, EC 1.15.1.1] activities were measured by inhibition of pyrogallol autoxidation by ethanol extracts of post-nuclear supernatants. Glutathione peroxidase [EC 1.11.1.9] activity of post-nuclear supernatants was measured by the procedure of Lawrence and Burk (1976). Glutathione concentrations of post-nuclear supernatants were measured with a kit from Cayman Chemical (Ann Arbor, MI). Protein concentrations were measured with the Folin phenol reagent [Peterson 1977].

Lipids were extracted from the subcellular fractions with a chloroform-methanol mixture [1.8:1, v/v] and were dried under nitrogen [Bligh and Dyer 1959]. The concentrations of conjugated dienes, products of lipid peroxidation formed in vivo, were measured by UV absorbance at 230 nm [Bacon et al. 1983] and were normalized to the total lipid content of extracts, which were quantified colorimetrically by reduction of potassium dichromate [Chiang et al. 1957].

Tissues were analyzed for manganese content using an atomic absorption spectrophotometer with a graphite furnace atomizer (Hitachi model 170-70 polarized Zeeman, Tokyo, Japan) as described previously [Davis et al. 1990]. Left tibias and kidneys were heated at 450°C in a muffle furnace and analyzed for iron by atomic absorption spectroscopy (model 3100, Perkin Elmer, Norwalk, CT). Tissues digested for manganese analysis were also analyzed for iron.

Fatty acid profile. Two additional rats per treatment were fed the diets for 6 wk, at which time they...
were killed and their heart and liver crude mitochondrial and crude microsomal fractions obtained by differential centrifugation as described above.

Extracted lipids from these subcellular fractions were evaporated under nitrogen, saponified and esterified ([Monsma and Ney 1993]). Fatty acid methyl esters were analyzed on a Varian 3400 gas chromatograph equipped with a flame-ionization detector, a Varian 1093 SPI programmable injector, a Supelcowax 10 column [Supelco, Bellefonte, PA] and an InBoard Data Handling System [IBDH, Sugarland, TX]. The relative quantities of fatty acids in samples were determined by comparing the peak areas from the gas chromatograms of samples to those of standards obtained from NuChek Prep [Elysian, MN], Matreya [Pleasant Gap, PA] and Sigma Chemical.

Diets were extracted and lipids were analyzed similarly. The fatty acid composition of the menhaden oil diets was as follows [mol/100 mol fatty acids]: 14:0 [9.7], 14:1 [0.3], 16:0 [23.4], 16:1 [11.8], 18:0 [3.3], 18:1 [13.0], 18:2 [5.4], 18:3 [1.3], 18:4 [3.4], 20:1 [1.0], 20:4 [0.8], 20:5 [16.9], 22:6 [9.7]. The corn oil diets contained the following [mol/100 mol fatty acids]: 14:0 [0.3], 16:0 [12.6], 16:1 [0.1],

**TABLE 1**

Conjugated diene formation in subcellular fractions of rats fed either menhaden oil or corn oil and either deficient or adequate concentrations of manganese

<table>
<thead>
<tr>
<th>Dietary treatment</th>
<th>Mitochondria</th>
<th>Microsome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heart</td>
<td>Liver</td>
</tr>
<tr>
<td></td>
<td>absorbance at 230 nm/mg lipid</td>
<td></td>
</tr>
<tr>
<td>Menhaden oil, -Mn</td>
<td>0.130 ± 0.008*</td>
<td>0.099 ± 0.014</td>
</tr>
<tr>
<td>Menhaden oil, +Mn</td>
<td>0.093 ± 0.006b</td>
<td>0.127 ± 0.030</td>
</tr>
<tr>
<td>Corn oil, -Mn</td>
<td>0.094 ± 0.001b</td>
<td>0.169 ± 0.058</td>
</tr>
<tr>
<td>Corn oil, +Mn</td>
<td>0.073 ± 0.006c</td>
<td>0.129 ± 0.023</td>
</tr>
</tbody>
</table>

Statistical effects determined by ANOVA

<table>
<thead>
<tr>
<th></th>
<th>Mitochondria</th>
<th>Microsome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat</td>
<td>0.001</td>
<td>NS²</td>
</tr>
<tr>
<td>Mn</td>
<td>0.001</td>
<td>NS</td>
</tr>
<tr>
<td>Fat times Mn</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM. Within a column, values that do not share a superscript letter are significantly different (P < 0.05); n = 4 pooled heart samples/treatment [2 hearts/pool]; n = 12 livers/treatment, except for the menhaden oil, manganese-deficient group, where n = 11 livers.

2 NS = not significant (P > 0.05).
18:0 (0.3), 18:1 (20.3), 18:2 (63.1), 18:3 (2.0), 20:0 (0.9), 20:1 (0.6).

**Statistical analysis.** The data were analyzed by two-way ANOVA (fat type and manganese level) using a SAS general linear model program (SAS 1985). Duncan's multiple range tests (SAS 1985) were used to differentiate between treatments. Values are reported as means ± SEM in the text.

**RESULTS**

Heart and liver manganese concentrations in the manganese-deficient rats were 56% of those in manganese-adequate rats, confirming manganese deficiency [Fig. 1]. Manganese was not lost equally from all subcellular fractions: manganese concentrations in liver mitochondria of manganese-deficient rats were 76% of those of manganese-adequate rats.

Manganese-deficient rats had more conjugated dienes in heart mitochondria than manganese-adequate rats [Table 1]. Manganese deficiency had no effect on conjugated diene formation in liver mitochondria, heart microsomes or liver microsomes. The type of dietary fat affected conjugated diene formation in heart mitochondria. Rats fed fish oil had more conjugated dienes in heart mitochondria than did those

![Graph](https://example.com/graph.png)

**FIGURE 2** Manganese superoxide dismutase (MnSOD) activities in mitochondria [panel a] and copper-zinc superoxide dismutase (CuZnSOD) activities in post-nuclear supernatants [panel b] of tissues of rats fed diets containing menhaden oil or corn oil and either deficient or adequate concentrations of manganese. Values are means with SEM. Within a tissue, values that do not share a superscript letter are significantly different [P < 0.05]. Manganese effects were significant in heart mitochondrial MnSOD activity [P < 0.0001] and heart CuZnSOD activity [P < 0.01]; n = 4 pooled heart samples/treatment [2 hearts/pool]; n = 12 livers/treatment, except for the group fed the menhaden oil, manganese-adequate diet, in which n = 11 livers.
Manganese-deficient rats had significantly (37%) less MnSOD activity in their heart than did manganese-adequate rats [Fig. 2a]. Manganese superoxide dismutase activities were inversely correlated with the concentration of conjugated dienes in heart mitochondria \( r = -0.71, P < 0.005 \). Manganese superoxide dismutase activity in liver was not affected by dietary treatment.

Copper-zinc superoxide dismutase activities were significantly (35%) greater in the hearts of manganese-deficient animals fed menhaden oil than in the hearts of manganese-adequate rats fed either oil [Fig. 2b]. Copper-zinc superoxide dismutase activity in liver was not affected by dietary treatment.

Dietary treatment did not affect heart or liver glutathione peroxidase activities or glutathione concentrations [Table 2]. Liver, but not heart, iron concentrations were slightly greater in manganese-deficient rats fed corn oil than in manganese-adequate rats fed fish oil. There was no effect of the type of dietary fat on tissue iron concentrations.

The type of dietary fat had no effect on manganese concentrations or enzyme activities, but affected lipid composition of heart and liver mitochondria [Table 3]. Fish oil consumption resulted in higher peroxidizability indices in mitochondria. In heart mitochondria, concentrations of 22:6 were higher in rats fed menhaden oil and concentrations of 18:3 and 20:4 were lower [based on 5 mol/100 mol difference between fat types]. Rats fed menhaden oil had higher concentrations of 16:0 and 22:6 in liver mitochondria, but lower concentrations of 18:0, 18:2 and 20:4. The type of dietary fat seemed to have less consistent effects on heart and liver microsomes. Manganese deficiency did not seem to affect lipid composition.

### TABLE 2

<table>
<thead>
<tr>
<th>Dietary treatment</th>
<th>Glutathione peroxidase (^2)</th>
<th>Glutathione concentration</th>
<th>Iron concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heart (\mu)mol/g protein</td>
<td>Liver (\mu)mol/g protein</td>
<td>Heart (\mu)mol/g wet wt</td>
</tr>
<tr>
<td>Menhaden oil, (-)Mn</td>
<td>59 ± 6</td>
<td>79 ± 3</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td>Menhaden oil, +Mn</td>
<td>58 ± 3</td>
<td>75 ± 4</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td>Corn oil, (-)Mn</td>
<td>70 ± 6</td>
<td>83 ± 3</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>Corn oil, +Mn</td>
<td>67 ± 4</td>
<td>79 ± 2</td>
<td>3.3 ± 0.2</td>
</tr>
</tbody>
</table>

\(1 \text{ Values are means} ± \text{ SEM. Within a column, values that do not share a superscript letter are significantly different}\ (P < 0.05), n = 4 \text{ pooled heart samples/treatment (2 hearts/pool), n = 12 livers/treatment, except for the menhaden oil, manganese-deficient group, where n = 11 livers.}

\(2 \text{ One unit of activity is defined as 1} \mu\text{mol of NADPH oxidized per minute.} \)
However, we observed only a slight elevation of liver iron in manganese-deficient rats.

Some, but not all, rat strains exhibit abnormal lipid metabolism in manganese deficiency (Davis et al. 1990, Klimis-Tavantzis et al. 1983). However, we showed that dietary manganese had no consistent effects on the lipid composition of heart and liver mitochondria and microsomes.

Thus, we eliminated many possible alternative mechanisms for the manganese antioxidant effect. Stepwise regression of the other antioxidant and prooxidant systems showed that only MnSOD activity was significant, and it explained 50% of variation in conjugated dienes.

**Effect of type of dietary fat.** Although dietary manganese had no effect on the lipid composition of heart and liver mitochondria and microsomes, the type of dietary fat did. Consumption of menhaden oil rather than corn oil resulted in higher peroxidizability indices in heart and liver mitochondria but not microsomes. This is consistent with the work of Kurato et al. (1994). This increase in highly unsaturated fatty acids in mitochondria of rats fed menhaden oil translated into higher concentrations of conjugated dienes in heart but not liver. There was no interaction effect between dietary manganese and type of dietary fat in conjugated diene concentrations in heart mitochondria. This suggests that dietary manganese and type of dietary fat have different but additive mechanisms affecting lipid peroxidation.

We observed no effect of fat type on MnSOD activities or other antioxidant enzymes. Several investigators (Davis et al. 1990, L’Abbé et al. 1991, Phylactos et al. 1994) observed differences in MnSOD activities with type of dietary fat, but in these studies tocopherol contents of the diets were not equal. Cho and Choi (1994) and Kaasgaard et al. (1992) also observed that normalizing vitamin E concentrations of diets attenuated differences in oxidative damage due to type of dietary fat.

We showed that, as expected, manganese deficiency reduced tissue manganese concentrations and, in at least one tissue, MnSOD activity. This loss of antioxidant in manganese-deficient animals could have functional consequences. Age-related declines in MnSOD activity have been related to increased lipid peroxidation and mitochondrial DNA deletions (Yen et al. 1993). The increased conjugated diene formation we noted in manganese-deficient rats may be related to observed mitochondrial ultrastructural changes (Hurley et al. 1970, Zidenberg-Cherr et al. 1985). More work should be done to relate conjugated diene formation and other evidence of lipid peroxidation to actual tissue damage in manganese deficiency.

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LITERATURE CITED


