Cyclo-Oxygenase Inhibition Restores the Attenuated Vasodilation in Manganese-Deficient Rat Aorta

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Abstract

Previously we showed that manganese (Mn) deficiency enhances the arterial contractile response to α1-adrenergic stimuli and affects vasomotor tone. The aim of this study was to test the hypothesis that dietary Mn deficiency inhibits the vasodilation pathways of rat aorta. Vascular ring studies were conducted in aortic rings from weaning male Sprague-Dawley rats that were fed either a Mn deficient (MnD) or a Mn adequate/control diet (MnA) (<1 and 12 mg/kg Mn, respectively) for a 14-wk period. We investigated endothelium-dependent vasodilation induced by acetylcholine (Ach; 10−8 to 3 × 10−6 mol/L) in isolated 3-mm aortic rings precontracted with L-phenylephrine (L-Phe; 10−6 mol/L). Seven concentrations of Ach were used in the presence or absence of inhibitors of nitric oxide synthase and cyclo-oxygenase. After a second precontraction, 8 doses of sodium nitroprusside (SNP; 10−8 to 10−6 mol/L) were added to assess endothelium-independent vasodilation. We observed a decrease in Ach-induced and SNP-induced vasodilation in MnD rat aortas when compared with MnA rat aortas (P < 0.05). Vessel sensitivity of MnD and MnA aortas to Ach was similar. The addition of L-arginine had no effect on nitric oxide–mediated vasodilation in either group. Nitric oxide synthase–inhibition blunted endothelium-dependent vasodilation to the same degree for both diet groups. Cyclo-oxygenase inhibition enhanced both Ach-induced and SNP-induced vasodilation of MnD rings compared with MnA aortic rings (P > 0.05). Manganese inhibits the synthesis or activity of a prostanoid-derived vasoconstrictor, which seems to be present at basal and at stimulated levels. This effect is independent of membrane-related events. Our results provide further information on the critical role of Mn on vasomotor tone. J. Nutr. 136: 2302–2307, 2006.

Introduction

The vascular endothelium regulates vascular tone by releasing endothelium-derived relaxing factors (EDRF)6 and endothelium-derived contracting factors (EDCF) (1–3). Nitric oxide (NO), prostacyclin (PGI2), and endothelium-derived hyperpolarizing factors are among the major relaxing factors. Shear stress and cell-membrane receptors activated by hormones and autacoids stimulate the release of NO from the endothelium by the formation of L-citrulline from L-arginine (L-Arg) (4). Nitric oxide induces vasodilation through the activation of cGMP-pathway (5), whereas prostacyclin, a product of cyclo-oxygenase, acts synergistically with NO to induce vasodilation (2,6). Finally, endothelium-derived hyperpolarizing factor induces vasodilation by acting on K+ ion channels (7).

The activation of specific receptors in the endothelial cell membrane by agonists such as acetylcholine (Ach) results in an increase of intracellular calcium, which activates nitric oxide synthase (NOS). L-Arginine is the physiological precursor for NO formation that requires NOS, as well as other cofactors (3). Nitric oxide has also been shown to interfere with endothelial cell-arachidonic acid pathways by stimulating the activity of cyclo-oxygenase (COX) in the production of eicosanoids (6,8,9). Nitric oxide seems to regulate the synthesis of eicosanoid vasodilators such as PGI2 as well as vasoconstrictors such as thromboxane A2 (TXA2) and prostaglandin H2 (PGH2). Alterations in the ratio between the vasoconstrictor and vasodilator eicosanoids are especially important for the development of vascular dysfunction (10).

With endothelial dysfunction there is an impairment of the vasodilator response, which involves multiple interacting signaling pathways. A decrease in the bioavailability of endothelium-derived vasodilators (i.e., NO) seems to be one of the primary risk factors for the development of several vascular diseases (11–16). Reduced NO bioavailability is the result of a decreased production (i.e., limited substrate availability; impaired NOS
activity or expression) or increased degradation of NO (17). Under oxidative stress, an interaction of superoxide with NO leads to the destruction of its vasodilator actions and the production of cytotoxic NO by-products, such as peroxynitrite (ONOO−). As a result, the signal transduction processes in which NO is implicated as a second messenger are obstructed. Impairment of this process is related not only to attenuated vasodilation but is also implicated in pathologic conditions such as hypertension (18), atherosclerosis (4), and diabetes (15).

Manganese participates as a cofactor in the structure of several enzymes and modulates important signal transduction pathways (19). Furthermore, Mn increases the accumulation of several enzymes (cAMP, cGMP) that activate proteins for further cell-signaling (20), modulates in vitro cell surface receptor binding and adhesion (21), and may function as a Ca++ ion entry blocker (20). Previously we reported that the presence of supplemented dietary Mn suppresses vasocostriction induced by an α1-adrenergic agonist (22). The effect of Mn on the regulation of vasomotor tone takes place through an endothelium-mediated pathway, possibly by affecting vasodilator tone (23,24) and/or by antagonizing Ca++ ions in the endothelium, thus impairing phenylephrine (Phe)-induced contractions in intact rings and potentiating vascular smooth muscle contractions (22). Few in vitro (25) and ex vivo studies (26) have suggested a role for Mn in endothelium-mediated vasodilation and there is limited information on its role on eicosanoid metabolism as it relates to vascular function. Thus, the goal of this study was to investigate the effect of dietary Mn on Ach-induced vasodilation (endothelium-dependent) and on sodium nitroprusside (SNP)-induced vasodilation (endothelium-independent) in male Sprague-Dawley rats fed a Mn deficient (MnD) or adequate (MnA) diet (27).

Materials and Methods

Animals. Thirty-two weaning male Sprague-Dawley rats (Charles River Laboratories) were randomly assigned to Mn-deficient (MnD; Mn < 1 mg/kg, n = 16) or Mn-adequate (MnA; Mn = 12 mg/kg, n = 16) diets upon arrival. The rats were individually housed in stainless steel cages in an environmentally controlled room (22 °C with a 12:12-h light-dark cycle). Body weights were measured weekly. The Animal Care and Use Committee of the University of Maine approved all animal care and experimental procedures.

Diets. Diets were mixed in our laboratory from purified ingredients, as described before (27,28). Vitamin (AOAC Special Vitamin Mixture, Harlan Teklad) and mineral mixtures (MMP Biochemicals) were commercially prepared. The Mn concentrations of diet preparations and tap water were measured by atomic absorption spectroscopy at the Maine Forest and Agriculture Experiment Station Analytical Laboratory, Plant and Soil Department of the University of Maine (detection limit 0.01 mg/kg or mg/L). Rats consumed food and water (tap water, below 6 mg/L of NaCl, KCl, NaHCO3, KH2PO4, MgSO4, 1.17; dextrose, 11; and CaCl2, 1.25). Liver and kidney tissues were weighed, lyophilized, pulverized, and analyzed for Mn using an atomic absorption spectrophotometer with a graphite furnace atomizer at the Maine Forest and Agriculture Experiment Station Analytical Laboratory, Plant and Soil Department of the University of Maine.

Tissue sampling. At the end of the feeding period (14 wk), food was withheld for 12–14 h. Rats were anesthetized in a chamber with 95% CO2 and 5% O2, for ~2 min. Thoracic aortas and livers were removed and washed with physiologic salt solution (PSS; with composition in mmol/L: NaCl, 118; KCl, 4.7; NaHCO3, 25; KH2PO4, 1.18; MgSO4, 1.17; dextrose, 11; and CaCl2, 1.25). Liver and kidney tissues were weighed, lyophilized, pulverized, and analyzed for Mn using an atomic absorption spectrophotometer with a graphite furnace atomizer at the Maine Forest and Agriculture Experiment Station Analytical Laboratory, Plant and Soil Department of the University of Maine.

Vascular ring studies. Thoracic aortas were removed carefully and submerged in a petri dish filled with PSS at room temperature. The aortic segment was cleaned of fat and connective tissue and divided into 4 rings of 3 mm length prepared from the middle section of the vessel. Each ring from each rat was used for 1 of 4 different treatments. As described before (22,29), rings were mounted in weightless triangles and placed in PSS (37°C, pH 7.4). Isometric force recordings were transferred to a tissue force analyzer (Model 410, MicroMed).

Experimental protocol. A total of 64 endothelium-intact rings from each diet group were used for the 4 different protocols. Four different protocols were applied for the 4 different ring treatments (4 rings from each rat, n = 16 rats). A resting tension of 1.5 g was applied during the precontraction and equilibration period (22,29,30).

One ring was washed with PSS without the addition of a substrate or an inhibitor. In a second ring, i-NMMA, a NOS L, II and III inhibitor (i-N6-monomethyl-arginine, 10−4 mol/L), was added to the bath for 10 min, and in a third ring, mephenemine acid (MFA; 10−5 mol/L) was added for 10 min as a COX I and II inhibitor. Both inhibitors remained in the tissue bath throughout the experiment. In a fourth ring, the NO substrate i-Arg (10−3 mol/L) was added to the tissue bath for 10 min to restore any reduction in dilation response due to a reduced availability of the NO substrate.

All aortic preparations were left to equilibrate for ~45 min and then all 4 rings were precontracted with 1 maximal dose of the α1 adrenergic agonist, i-Phe (10−6 mol/L), for 10 min, which was the duration necessary for the contraction curve to reach a plateau. Following the i-Phe precontraction, cumulative applications of 6 concentrations of Ach (10−8 to 3 × 10−6 mol/L) were applied, allowing a drug-tissue contact time of 6 min, during which maximum vasodilation was achieved.

Aortic rings were washed 4 times over a 20 min period with aerated PSS (37°C, pH 7.4) and the substrate (i-Arg) and the 2 inhibitors (i-NMMA or MFA) were added back to the same tissue baths. Rings were allowed to equilibrate to baseline to bring aortic tension to the initial preload level. Endothelium-independent dilation was then assessed with 7 doses of sodium nitroprusside (SNP; 10−6 to 10−5 mol/L), as previously described (30); SNP is a NO donor and a cGMP-dependent vasodilator. A drug-tissue contact time of 4 min was allowed for each SNP concentration to achieve the maximum dilation for each dose. One dose of the nonselective and endothelium-independent vasodilator papaverine (10−3 mol/L) was used in the end to obtain maximal vasodilation. Papaverine is a nonspecific inhibitor of phosphodiesterase in the vascular smooth muscle; it increases cAMP and cGMP concentrations and indirectly induces vasodilation.

The relaxant effect to each agonist dose was expressed as a percentage of vasodilation to the initial i-Phe precontraction. The EC50 values were determined for each ring (the effective concentration of agonist in which 50% of maximum response was obtained). The negative log (base 10) of the EC50 value was calculated. Concentration response curves were fitted by nonlinear regression. The pD2 (−logEC50) was calculated to evaluate the vessel sensitivity to the agonists. The pD2 values for aortic ring responses to agonists (Ach and SNP) were compared among diet groups.

Drugs and chemicals. Acetylcholine chloride, sodium nitroprusside, i-phenylephrine, i-N6-monomethyl-arginine, i-arginine, mephenemine acid, papaverine, and salts for the stock solutions of the physiologic salt solution (PSS: NaCl, KCl, NaHCO3, KH2PO4, MgSO4, Dextrose, CaCl2) were purchased in pure forms from Sigma-Aldrich.

Statistical analysis. All results were expressed as means ± SEM; tests were performed with α = 0.05. Rat body, liver, and kidney weights and hepatic and kidney Mn concentrations were compared between diet groups using a 1-way ANOVA. Changes in vasodilation and pD2 values due to agonists were compared by 2-way ANOVA and post hoc Student-Newman-Keuls pairwise multiple-comparison procedure to determine the effect of the diets on maximum vasodilation and on vessel sensitivity. The statistical program used was the Sigmapstat Statistical Program Package, version 2.0 (SPSS).
Results

Animal growth. Rats fed either MnD or MnA diet gained weight (Table 1); the groups differed in growth rate beginning at wk 4, as was observed in our previous experiments with MnD rats (22,28). The differences in liver weights among diet groups (Table 1) were proportionate to the differences in body weight as indicated by similar liver weight to body weight ratios (Table 1), an indicator of normal growth. Manganese deficiency was confirmed by hepatic and kidney Mn concentrations, which were lower in the MnD group (Table 1). The mean daily food intake was measured in previous experiments (22,28) and did not differ among rats from different diet groups. Thus, food intake was not measured in this experiment.

Endothelium-dependent vasodilation. Six cumulative doses were used to give Ach concentrations of 10^{-8} to 3 \times 10^{-6} \text{mol/L} in 3-fold steps in the tissue baths. Each dose produced a graded decrease of the maximum l-Phè-induced precontraction force, which was considered to be the point of 0% vasodilation (percentage of the level of precontraction). At the end of the cumulative Ach dilation curve, the force returned to a lower level than its initial passive tension (preload). The baseline point (preload, 0 g of force developed) was considered to be 100% dilation. Washout of agonists 4 times after the end of the protocol allowed the force to return to at least the preload value. Acetylcholine-induced vasodilation was lower in the MnD diet group than in the MnA group (Fig. 1A). The maximum percentage of vasodilation was significantly lower in the MnD group than in the MnA group.

The pD₂ values for aortic ring dilations to Ach, a measure of vessel sensitivity to the Ach receptor response, were calculated using EC_{50} values (effective concentration of Ach in which 50% of maximum dilation was obtained) and did not differ between MnD (8.33 ± 0.07) and MnA (8.39 ± 0.06) groups. The vasodilation to the one dose of papaverine did not differ between diet groups.

Endothelium-independent vasodilation. The maximum percentage of vasodilation in response to SNP was significantly lower in MnD group than in the MnA group (Fig. 1B). However, vessel sensitivity to SNP did not differ between diet groups (MnD: 8.41 ± 0.06 vs. MnA: 8.38 ± 0.07).

Vasodilation after in vitro supplementation with L-arginine. Pretreatment of the rings with the NOS substrate L-Arg did not reverse the previously observed decreases in Ach-induced vasodilation in the MnD group (Fig. 1A). Thus, L-Arg is not a limiting factor for NO synthesis in MnD rats because the addition of the substrate did not affect the maximum vasodilation in MnD aortic rings (127% before vs. 125% after the addition of L-Arg). This suggests that Mn deficiency in rats does not affect L-Arg availability in the aorta.

Endothelium-dependent vasodilation in the presence of inhibitors. The addition of L-NMMA reduced the dilator response to Ach in both diet groups (Fig. 2A). The reductions in vasodilation after treatment with L-NMMA were similar between diet groups. The vessel sensitivity to Ach was reduced in the presence of L-NMMA but did not differ between diet groups (MnD: 7.56 ± 0.06 vs. MnA: 7.58 ± 0.1).

In MnD rats treated with the COX inhibitor MFA, we observed an increased response to Ach-induced vasodilation compared to rings with no inhibitor (Fig. 2B). This increase in dilation in the MnD group restored Ach-induced dilation to that of the MnA group (Fig. 2B). Despite the augmented dilator response in MnD rings treated with MFA, the vessel sensitivity to Ach in the presence of MFA did not differ between diet groups (MnD: 846 ± 0.08 vs. MnA: 8.32 ± 0.07).

Endothelium-independent vasodilation in the presence of a COX inhibitor. In the aortic rings of MnD rats treated with the COX inhibitor, we observed an increased maximum response to SNP-induced vasodilation (Fig. 3). Similarly to the Ach-induced dilation, MFA restored SNP-induced dilation in the MnD group to levels equal to those of the control group (Fig. 3). The addition of MFA affected only the response to SNP in MnD rats but had no effect on MnA rats.

Discussion

We have previously documented that Mn is important for the contractile machinery during vasoconstriction induced by an \( \alpha_1 \) adrenergic agonist ex vivo (22–24). Our studies supported the hypothesis that Mn can pass through voltage-dependent Ca^{2+} channels and might share a Ca^{2+}-blocking property in smooth muscle cells by antagonizing Ca^{2+} entry (31–34). The present study demonstrates that Mn deficiency attenuates endothelium-dependent vasodilation (induced by Ach) and endothelium-independent vasodilation (induced by SNP) (Fig. 1). Manganese seems to function primarily independent of NOS, NO substrate (L-Arg) availability, and vessel sensitivity to the agonist (Ach), because a diet deficient in Mn did not alter the NO-mediated component of Ach-induced vasodilation. Our results further demonstrated that the attenuated vasodilation to both Ach and SNP does not occur when COX (1 and/or 2) is inhibited, indicating the synthesis or activation of a COX-dependent vasoconstrictor prostanoid in aortas of MnD rats. Thus, Mn deficiency seems to influence the primary end product of the endothelial COX-mediated arachidonic acid metabolism and possibly reduce the bioactivity of endothelium-generated vasodilators without affecting membrane-related events.

Ensunsa et al. (26) suggested that Mn deficiency is characterized by increased vasodilation, which may be attributed to an increased availability of NOS and NO production due to the attenuated activity of arginase (the enzyme that competes with eNOS for the substrate L-Arg). However, this and previous studies reported a reduced liver and kidney arginase activity in rats fed MnD diets when compared with control diets, but vascular arginase activity was not measured (26,35,36). Additionally, experimental diets from the above studies contained

TABLE 1 Effects of dietary Mn on body, liver, and kidney weights and on liver and kidney Mn concentrations

<table>
<thead>
<tr>
<th>Diet groups</th>
<th>Body wt, g</th>
<th>Liver wt, g</th>
<th>Liver wt/body wt</th>
<th>Liver Mn, ( \mu g/g )</th>
<th>Kidney wt, g</th>
<th>Kidney Mn, ( \mu g/g )</th>
</tr>
</thead>
<tbody>
<tr>
<td>MnD</td>
<td>462 ± 12 ±</td>
<td>13.57 ± 0.58*</td>
<td>2.9 ± 0.001</td>
<td>1.22 ± 0.05*</td>
<td>3.45 ± 0.01</td>
<td>1.01 ± 0.07*</td>
</tr>
<tr>
<td>MnA</td>
<td>526 ± 22</td>
<td>16.09 ± 0.93</td>
<td>3.0 ± 0.001</td>
<td>5.48 ± 0.19</td>
<td>3.50 ± 0.1</td>
<td>2.79 ± 0.06</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, \( n = 16 \). *Different from MnA, \( P \leq 0.05 \).
2 To convert μg/g of liver and kidney Mn to μmol/g, divide by 54.938.
much higher concentrations of Mn (45, 48, and 50 mg/kg) than our control diet (12 mg/kg, which was set by the AIN as the amount that supports good growth in rats with no signs of deficiency) (27). Our experimental data, at the level of Mn studied, do not support the hypothesis that arginase deficiency in rat aortic tissue affects endothelium-dependent vasodilation, especially because we did not directly measure vascular arginase activity.

We showed that COX-inhibition restores the endothelium-dependent and -independent vasodilation in Mn deficiency, which suggests an enhanced production of a COX-dependent vasoconstrictor. The presence of reactive oxygen species has been shown to favor the production of vasoconstrictor (TXA₂) instead of vasodilator prostanoids (PGI₂) (10). Bovine aortic endothelial cells have been reported to produce higher TXA₂ (vasoconstrictor) and lower PGI₂ (vasodilator) in Se deficiency, which has been associated with aging, but most importantly is a pathological indicator in children and adolescents with a predisposition to coronary heart disease (37,38). Manganese, due to its role as an essential cofactor for manganese superoxide dismutase (MnSOD), is considered to be one of the important antioxidant defense mechanisms in the cellular environment (39). Deficiency of Mn reduces the activity of MnSOD in liver, heart, and kidney, allowing the formation of superoxide (26,40,41). The accumulation of reactive oxygen species in the case of reduced activity of MnSOD might have profound effects on the production of eicosanoids through the COX pathway, as previously indicated for other trace mineral deficiencies such as selenium (Se) (42). In future studies, direct measurements on the production of vasoconstrictor and vasodilator prostanoids are necessary to confirm this hypothesis.

Our data suggest that Mn deficiency shifts the balance of endothelium-derived prostanoids by leading to a basal or stimulated release of an endothelium-derived vasoconstrictor, such as PGH₂ or TXA₂, possibly by inhibiting prostacyclin synthase. In spontaneously hypertensive rats, an impairment of endothelium-dependent vasodilation was associated with an increased release of TXA₂, a vasoconstrictor prostanoid underlying the endothelial cell dysfunction in hypertension (43). Cyclo-oxygenase inhibition increased the response to Ach in hypertensive rats with no effect on Ach-induced vasodilation in normotensive rats (10). Additionally, COX derivatives can curtail endothelial responses in essential hypertension (44). Even though there is limited information on the mechanisms underlying the modifications in prostanoid pathway by the presence of dietary Mn, we do know that patients with congestive heart failure as the result of ischemic heart disease or cardiomyopathy have 82.8% insufficient intake of dietary Mn (45), whereas patients with atherosclerosis appear to have decreased concentrations of Mn in aorta, liver, myocardium, adrenal glands, pancreas, and kidney when compared with healthy individuals (46). Thus, the presence of Mn might modulate the synthesis or activity of key enzymes in the complex COX pathway. In Se deficiency, accompanied by increased oxidative stress, the activity of PGI₂ synthase is downregulated, which might be the case in Mn deficiency as well (42).

In conclusion, a diet deficient in Mn substantially enhances arterial contractile response to α₁ adrenergic stimuli and promotes the synthesis or activity of a prostanoid-derived vasoconstrictor under basal or stimulated conditions. The presence of oxidative stress in Mn deficiency due to the reduced activity of the production of vasoconstrictor and vasodilator prostanoids is necessary to confirm this hypothesis.

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In conclusion, a diet deficient in Mn substantially enhances arterial contractile response to α₁ adrenergic stimuli and promotes the synthesis or activity of a prostanoid-derived vasoconstrictor under basal or stimulated conditions. The presence of oxidative stress in Mn deficiency due to the reduced activity of the
major antioxidant enzyme MnSOD might decrease endothelium-dependent and -independent vasodilation by enhancing the production of endothelium-derived vasoconstrictors such as Pgh2 or TxA2, with the concomitant inhibition of endothelium-derived vasodilators such as PGI2. In the U.S., intake of Mn may be compromised because of the routine use of supplements, such as calcium. In addition, high intakes of processed foods and refined carbohydrates might reduce the absorption or retention of Mn (47,48). Suboptimal intakes of Mn have been also documented in a number of diseases such as epilepsy, rheumatoid arthritis, multiple sclerosis, and asthma (49–52). Our findings provide further evidence for the critical role of Mn and its effects on vascular function and suggest a therapeutic role for Mn supplementation in populations with suboptimal Mn intake and in populations with a high risk for cardiovascular disease.

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Literature Cited
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