Reducing Arginase Activity *via* Dietary Manganese Deficiency Enhances Endothelium-Dependent Vasorelaxation of Rat Aorta

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L-Arginine is a common substrate for the enzymes arginase and nitric oxide synthase (NOS). Acute inhibition of arginase enzyme activity improves endothelium-dependent vasorelaxation, presumably by increasing availability of substrate for NOS. Arginase is activated by manganese (Mn), and the consumption of a Mn-deficient (Mn−) diet can result in low arginase activity. We hypothesize that endothelium-dependent vasorelaxation is greater in rats fed Mn− versus Mn sufficient (Mn+) diets. Newly weaned rats fed Mn− diets (0.5 μg Mn/g; $n = 12$) versus Mn+ diets (45 μg Mn/g; $n = 12$) for 44 ± 3 days had (i) lower liver and kidney Mn and arginase activity ($P \leq 0.05$), (ii) higher plasma L-arginine ($P \leq 0.05$), (iii) similar plasma and urine nitrate + nitrite, and (iv) similar staining for endothelial nitric oxide synthase in thoracic aorta. Vascular reactivity of thoracic aorta (~720 μm i.d.) and small coronary arteries (~110 μm i.d.) was evaluated using wire myographs. Acetylcholine (ACh; 10^{-8}–10^{-4} *M*) produced greater ($P \leq 0.05$) vasorelaxation in thoracic aorta from Mn− rats (e.g., maximal percent relaxation, 79 ± 7%) versus Mn+ rats (e.g., maximal percent relaxation, 54 ± 9%) at 5 of 7 evaluated doses. Tension produced by NOS inhibition using N^G monomethyl-L-arginine (L-NMMA; 10^{-3} *M*) and vasorelaxation evoked by (i) arginase inhibition using difluoromethylornithine (DFMO; 10^{-7} *M*), (ii) ACh (10^{-8}–10^{-4} *M*) in the presence of DFMO, and (iii) sodium nitroprusside (10^{-9}–10^{-4} *M*) were unaffected by diet. No differences existed between groups concerning these responses in small coronary arteries. These findings support our hypothesis that endothelium-dependent vasorelaxation is greater in aortic segments from rats that consume Mn− versus Mn+ diets; however, responses from small coronary arteries were unaffected. Exp Biol Med 229:1143–1153, 2004

Key words: nitric oxide; nitric oxide synthase; difluoromethylornithine; superoxide dismutase; nitrotyrosine

L-Arginine is a common substrate for the enzymes arginase, nitric oxide synthase (NOS), arginine decarboxylase, arginine:glycine amidinotransferase, and arginyl-tRNA synthetase (1). Of these enzymes, arginase and NOS have generated considerable interest regarding their interplay in the metabolism of L-arginine (2, 3). Arginase hydrolyzes L-arginine to form urea and ornithine. This process is important for removing ammonia from the body and for synthesizing proline and glutamate. Nitric oxide synthase catalyzes the hydrolysis of L-arginine to form nitric oxide (NO) and L-citrulline. It is now well accepted that NO produced in the vascular endothelium is a critical regulator of vascular tone and peripheral resistance.

Arginase can regulate L-arginine concentrations to an extent whereby the production of NO is influenced (2, 4, 5). Evidence to support this statement is that intravenous arginase infusion can induce cerebral arteriole constriction (5), while acute arginase inhibition can enhance coronary arteriolar vasodilation (4). These data suggest that acute increases or decreases of arginase activity can increase or decrease vascular tone, respectively. A mechanism potentially responsible for increased vasodilation in the presence of arginase inhibition is that more L-arginine is made available for NOS, resulting in increased NO production.

Arginase is a manganese (Mn)-containing enzyme that exists in two isoforms (6, 7). The predominant isoform in the liver is arginase I, and arginase II is most prominent in the kidneys and throughout the body (8). It has been shown that arginase I is constitutively present in rat aortic endothelial cells and arginase II can be induced by cytokine activity in

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this cell type (9). Studies show that diet-induced Mn deficiency can lower liver and kidney arginase activity in rats (10, 11). Although reducing arginase activity *via* Mn deficiency can result in higher ammonia levels and lower urea levels in the plasma (12), nondetrimental effects have also been reported. For example, preserved glomerular filtration rate and lower blood pressure were observed in rats with chronic renal failure that were fed Mn-deficient (Mn–) versus Mn-sufficient (Mn+) diets (11). The authors attribute these latter findings to improved NO production by endothelial cells in the Mn– animals (11). Because preserved glomerular filtration rate and lower blood pressure can result, in part, from arteriolar vasodilation, we sought to determine whether long-term Mn deficiency reduces arginase activity to an extent whereby endothelium-dependent vasorelaxation is improved. Specifically, we tested the hypotheses that endothelium-dependent vasorelaxation is increased in thoracic aorta and small coronary arteries from rats fed an Mn– diet relative to that observed in rats fed an Mn+ diet.

Materials and Methods

Experimental Animals and Diets. The experimental protocol was approved by the University of California at Davis Animal Use and Care Administrative Committee. Rats were housed in suspended stainless steel cages in a temperature-controlled (22°C) and light-controlled (12:12-hr light:dark) room. Pregnant Sprague-Dawley rats were obtained from a commercial vendor (Charles River Laboratories, Wilmington, MA). After their arrival (gestation Day 18), all dams were given a purified Mn– diet (0.5 μg Mn/g). Following delivery, dams and litters continued to receive the Mn– diet until the pups were weaned at postnatal Day 20. During this time, pups were allowed free access to the diet. At postnatal Day 20, female offspring were separated into two groups. Female rats were used because they express higher levels of endothelial nitric oxide synthase (eNOS) than males (13, 14). Thus, we rationalized that using females could result in greater differences in arginine flux through the NOS pathway(s) as a result of reducing arginase activity due to Mn deficiency. One group was given the purified Mn– diet while the other was given a purified Mn+ diet (i.e., control; 45 μg Mn/g). A detailed description of each diet has been published (15). At postnatal Day 63, rats were transferred to metabolic cages and urine was collected over 12 hours for measurement of nitrate + nitrite and creatinine (see Plasma and Urine Analyses). Because preliminary results indicated body weights to be lower in Mn– versus Mn+ animals, food intake and body weight were monitored from postnatal Days 60 to 64 in a subgroup of animals.

At postnatal Day 64, rats were anesthetized with ketamine (30–50 mg/kg im) and xylazine (3–5 mg/kg). The caudal artery was cannulated to measure systemic pressure and obtain blood samples (see Plasma and Urine Analyses; Refs. 16–18). Next, the chest was opened and the heart was excised and placed in ice-cold normal physiologic saline solution (NPSS; see Measurement of Vascular Reactivity). The liver, kidney, and apical portion of the heart were rapidly excised, immediately placed in liquid nitrogen, and stored at –80°C (see Tissue Analyses). Segments of thoracic aorta were dissected carefully from surrounding tissue, placed in ice-cold NPSS, and used to determine vascular reactivity. In a subgroup of animals from each group, entire hearts were removed and frozen at –80°C (see Tissue Analyses) and sections of aorta were fixed in 4% paraformaldehyde in phosphate-buffered saline at pH 7.4 (see Immunohistochemical Analyses).

Measurement of Vascular Reactivity: Thoracic Aorta. The thoracic aorta was immersed in ice-cold NPSS (pH ~7.4) and trimmed carefully of connective tissue, and four segments were mounted on individual wire-type myographs (Jules Osher, Pomona, CA). Two tungsten wires (o.d. = 40 μm) were inserted in a parallel manner through the lumen of the vessel. One wire was attached to a force transducer (Fort10 Transducer; World Precision Instruments, Sarasota, FL) to measure tension development, while the other was fixed to a micrometer that was used to stretch the vessel in small increments. Vessels were immersed in a temperature-controlled, 8.5-ml reservoir (i.e., a tissue bath) containing oxygenated (95% O_2 and 5% CO_2) NPSS (pH ~7.4). Samples from all buffers and each tissue bath were analyzed frequently for P_{O_2}, P_{CO_2}, and pH. After the arteries were mounted, the tissue bath was gradually warmed to 37°C and the vessels were equilibrated at 2 *g* of tension for 60 mins (19). During this time, contents of the vessel chamber were exchanged at 10- to 15-min intervals with fresh oxygenated NPSS.

Experiments were separated by 30 mins and performed on 18 to 24 vessels per group that were precontracted with norepinephrine (NE; 10^{-7} *M*; Ref. 19). Vasorelaxation responses to acetylcholine (ACh; 10^{-8}–10^{-4} *M*), sodium nitroprusside (SNP; 10^{-9}–10^{-4} *M*), difluoromethylornithine (DFMO; 10^{-7} *M*), and ACh (10^{-8}–10^{-4} *M*) in the presence of DFMO (10^{-7} *M*), and vasocontraction produced by N^G monomethyl-L-arginine (L-NMMA; 10^{-3} *M*) were recorded.

Coronary Arteries. With the heart immersed in ice-cold NPSS, second- and third-order branches from the left coronary artery were isolated, removed, and prepared for mounting on the microvessel myograph. Procedures were the same as those described for aortic segments except that 20 μm (o.d.) tungsten wires were inserted through the lumen. After the coronary arteries were mounted, the tissue bath was gradually warmed to 37°C and the vessels were equilibrated at 10 mg of tension for ~30 mins. During this period, the vessel-bathing medium was exchanged at 10- to 15-min intervals with fresh, oxygenated NPSS. Next, internal circumference-active tension curves were constructed to determine the artery diameter that evoked the greatest tension development (i.e., L_{max}) to 100 m*M* potassium chloride (KCl). L_{max} was determined for every vessel, and this tension was maintained throughout the study. After a second 30-min equilibration period, the same series of experiments as described for the aorta was

performed except that vessels were precontracted with KCl (45 m*M*; $n = 9$–12 vessels/group) or endothelin-1 (ET-1; ~3×10^{-8} *M*; $n = 9$–12 vessels/group). Potassium chloride was used because it negates the potential contribution to relaxation from endothelium-derived hyperpolarizing factor (EDHF). This was an important consideration because we were primarily interested in whether reducing arginase activity could increase NO-mediated vasorelaxation. However, because EDHF has been shown to contribute to ACh-evoked relaxation in canine coronary microvessels (20) and rat mesenteric arteries (21) and a contribution from non–NO-dependent mechanisms to ACh-induced vasorelaxation of "resistance sized" vessels is becoming increasingly evident (22), we also tested our hypotheses in vessels precontracted with ET-1.

For all protocols involving aortic segments and coronary arteries, the appropriate time/volume/vehicle controls were performed. For example, when precontraction tension was stable, an appropriate volume of the inactive enantiomer (e.g., D-NAME for L-NAME) or vehicle (e.g., double-distilled water for ACh) was administered to the vessel-bathing medium while tension was monitored for a duration similar to that required for the dose-response curve (e.g., ~5 mins for evaluation of basal NO-synthase activity or ~10 mins for ACh-evoked vasorelaxation). In all cases, no significant changes from the original tension were observed, verifying the stability of precontraction regardless of time, vehicle, or volume (these standard controls were used in all studies). Further, the efficacy of NO-synthase inhibition after L-NMMA was tested by administering 10^{-4} *M* ACh in all cases. All tension data were continuously recorded by a computer through an analog-to-digital interface card (Biopac Systems Inc., Santa Barbara, CA) that allowed for subsequent off-line quantitative analyses. We have previously used these methods (16–18, 23).

Vasocontractile responses for both vessel types are presented as mg of developed tension (i.e., NE, KCl, ET-1) or percent increase from precontraction tension (L-NMMA). Vasorelaxation responses (i.e., ACh, SNP, DFMO, DFMO + ACh) are expressed as percent relaxation from precontraction tension.

Plasma and Urine Analyses. To measure arginine, plasma samples were first deproteinized with an equal volume of 6% (w/v) sulfosalicylic acid and centrifuged at 14,000 *g* for 25 mins at 4°C. The supernates were injected onto a Beckman 6300 amino acid analyzer (Beckman Coulter Inc., Fullerton, CA).

For nitrate + nitrite analysis, plasma previously collected into EDTA-containing tubes was centrifuged at 1800 *g* for 15 mins at 4°C before –80°C storage. Twelve-hour urine samples were similarly stored. Plasma and urine nitrate + nitrite were analyzed using a modified method of the Griess reaction (24, 25), while urine creatinine was measured using a commercially available kit that utilizes a modified colorimetric method of Jaffe (diagnostic kit #555-A; Sigma Chemical Co., St. Louis, MO).

Tissue Analyses. Arginase activity was evaluated in segments of frozen liver (to estimate arginase I activity) and kidney (to estimate arginase II activity) that were homogenized in 0.1% (w/v) hexadecyltrimethylammonium bromide (9 vol/g tissue) and centrifuged at 4000 *g* for 15 mins at 4°C (26). The supernatant fractions were removed, diluted with water (1:9 for liver; 1:2.5 for kidney), and incubated with an equal volume of 100 m*M* sodium glycinate buffer, pH 9.5, with 50 m*M* arginine (500 µl total volume) for either 10 or 30 mins (liver or kidney, respectively) at 37°C. Urea standards prepared in water (27) were run simultaneously. The reaction was stopped by adding 2 ml of 0.1 *N* perchloric acid, and 25 µl of the acidified sample was reacted with 2.5 ml of antipyrine/diacetylmonoxime reagent (16 m*M* antipyrine [2,3-dimethyl-phenyl-3-pyrazolin-5-one], 50 m*M* diacetylmonoxime [2,3-butanedione monoxime], 43.5 m*M* arsenic[V] oxide, and 2.8% [v/v] sulfuric acid) for 15 mins at 100°C. The resulting solution was cooled in an ice water bath and analyzed spectrophotometrically within 15 mins using a Wallac Victor^2 1420 plate reader (Perkin Elmer Wallac, Gaithersburg, MD) at a wavelength of 450 nm. An aliquot of the supernatant fraction was assayed for total protein (28) using Bio-Rad protein reagent (Bio-Rad Laboratories, Hercules, CA) and bovine serum albumin as the standard. Several attempts were made to quantify arginase activity in the heart, aorta, and coronary arteries, but values obtained were below the assay detection level.

Superoxide dismutase (SOD) activity was measured in frozen liver, kidney, and heart. Tissue was homogenized in Tris and sucrose buffer (10 m*M* Tris, 0.25 *M* sucrose, pH 7.4; 9 vol/g tissue). Homogenates were sonicated to disrupt the mitochondrial membranes and centrifuged at 10,000 *g* for 30 mins at 4°C, and the supernates were used for analysis. Total SOD (copper and zinc superoxide dismutase [CuZnSOD] + manganese superoxide dismutase [MnSOD]) and MnSOD activities were measured (29) where 1 unit of enzyme activity was defined as the amount of enzyme required to inhibit the autoxidation of pyrogallol by 50%. The CuZnSOD was calculated as the difference between total and MnSOD. Protein concentrations were determined for the supernates as described earlier. The SOD activity was not measured in vessel tissue because the amount that remained after studies described earlier was limited and not sufficient for detection by this assay.

For measuring trace elements, portions of liver, kidney, and heart (~0.5 g) were placed in Erlenmeyer flasks and wet-ashed in 5 ml of concentrated nitric acid (12 *M*) at 70°C. The digested samples were then quantitatively transferred to volumetric flasks and analyzed for Mn, zinc, copper, and iron on a TJA Video 12 atomic absorption spectrophotometer by inductively coupled plasma atomic emission spectroscopy (Thermo Jarrell Ash Corp., Franklin, MA; Ref. 30).

Immunohistochemical Analyses. Aortae fixed in 4% paraformaldehyde were dehydrated through a series of ethanol washes, cleared in toluene, and embedded in

paraffin. Transverse sections (7–8 μm, ~1 cell layer) were cut and mounted on slides. Slide-mounted sections were then deparaffinized in xylene and rehydrated through a series of ethanol washes. Slides were incubated in 0.6% H_2O_2 for 30 mins at room temperature to block for endogenous peroxidase activity, digested for 7 mins at 37°C in 0.05% trypsin to expose the antigens, and incubated in 10% goat serum for 1 hr at room temperature to block nonspecific-protein binding. The primary antibodies for eNOS (BD Biosciences, Lexington, KY) and nitrotyrosine (NT; Upstate, Waltham, MA) (both rabbit polyclonals) were applied to separate sections overnight at 4°C at the following dilutions: eNOS 1:250; NT 1:1000. All primary antibodies were diluted in the appropriate blocking serum. The following day, slides were incubated for 2 hrs at room temperature with a biotinylated secondary antibody. Localization and expression of the specific proteins were visualized with an avidin-horseradish peroxidase-staining system (ABC kit; Vector Laboratories, Burlingame, CA) in the presence of 3,3-diaminobenzidine and H_2O_2. Sections were counterstained with Toluidine blue and examined for staining by light microscopy. For all experiments, one section on each slide did not have antibody applied and thereby served as a negative control. For each protein, the immunoassay was performed on individual samples ($n = 6$) in triplicate. All sections were scanned, coded (de-identified), and examined for intensity and pattern of staining by blinded evaluators. Staining intensity of images was ranked using an arbitrary scale (1–5, where 1 = an image with little or no staining and 5 = an image with intense staining).

Drugs and Solutions. All chemicals were obtained from Sigma Chemical except for DFMO (Calbiochem, San Diego, CA). Normal physiologic saline solution contained NaCl (125 m*M*), KCl (4.7 m*M*), KH_2PO_4 (1.2 m*M*), $MgSO_4$ (1.2 m*M*), $CaCl_2$ (2.5 m*M*), $NaHCO_3$ (18 m*M*), Na_2EDTA (0.026 m*M*), and glucose (11.2 m*M*). Acetylcholine, SNP, L-NMMA, NE, and DFMO were prepared daily from stock solutions using distilled de-ionized water.

Statistical Analyses. Vessel characteristics, L-NMMA–evoked vasocontraction, and plasma, urine, and tissue analyses (including immunohistochemistry scores) were compared between groups using an unpaired *t* test. Acetylcholine, SNP, and ACh + DFMO concentration-relaxation curves were compared using a two-way (dose vs. experimental group), repeated-measures analysis of variance. This identified differences among doses within each group and between groups at each dose. When a significant *P* value was obtained (i.e., $P \leq 0.05$), a Tukey *post-hoc* test was used to determine where the difference(s) existed (31). Results are presented as mean ± standard error of the mean.

Results

Although animals consumed their respective diets for similar durations (i.e. 44 ± 3 days), body weight at postnatal Day 64 was lower ($P \leq 0.05$) in Mn– rats (182 ± 6 g) versus Mn+ rats (211 ± 5 g). Because we identified this trend prior to completing the study, body weight and food intake were recorded from postnatal Days 60 to 64 in a subset of animals ($n = 6$ per group). During this time, no differences were noted between groups with respect to average daily food intake (15 ± 1 g/day) or food efficiency measured as Δ body weight/Δ food intake (0.15 ± 0.02 g body weight/g food intake daily). Liver and kidney weights were similar between groups (data not shown). Mean arterial pressure was not different between Mn– animals (90 ± 5 mm Hg) and Mn+ animals (94 ± 10 mm Hg).

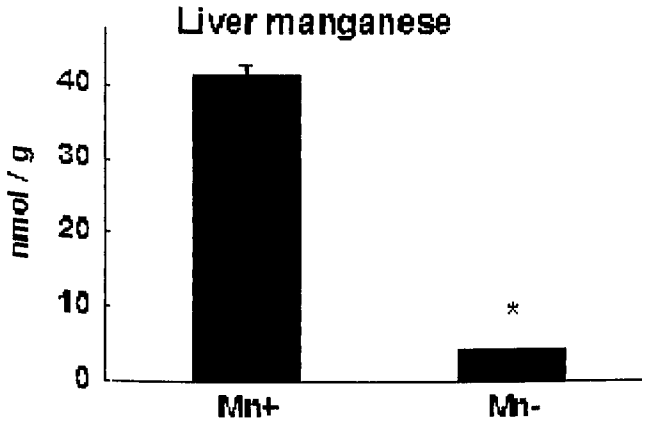
Enzyme and Mineral Analyses. Liver, kidney, and heart Mn concentrations were lower ($P \leq 0.05$) in Mn– versus Mn+ animals by ~10-fold (Fig. 1). Manganese concentrations in Mn+ animals were similar to those in rats that consumed diets containing trace element levels based on the National Research Council's recommendations for laboratory animals (10, 32). Zinc, copper, and iron concentrations were similar in the two groups (data not shown). Liver and kidney arginase activities were 61% and 54% lower ($P \leq 0.05$), respectively, in Mn– rats than in the Mn+ animals (Fig. 2). The MnSOD activities were lower ($P \leq 0.05$) in the liver, kidney, and heart of the Mn– rats than in the Mn+ rats. The CuZnSOD activities were similar between groups (Table 1).

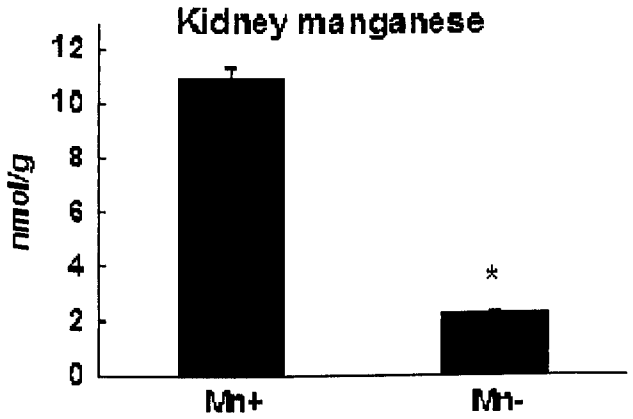
Vascular Reactivity. Vessel characteristics were similar between groups for the aortic segments and coronary arteries (Table 2).

Acetylcholine-evoked vasorelaxation was greater ($P \leq 0.05$) in segments of thoracic aorta from Mn– versus Mn+ animals (Fig. 3, Panel A). Baseline tension (~1995 mg) and precontraction tension developed in response to 10^{-7} NE (~822 mg) were similar between groups before adding ACh. Sodium nitroprusside–induced responses were not different between vessels from Mn– and Mn+ animals (Fig. 3, Panel B). Baseline tension (~1996 mg) and precontraction tension developed in response to 10^{-7} NE (~784 mg) were similar between groups prior to administering SNP.

The L-NMMA–induced vasocontraction was not different between Mn– animals (111 ± 24%) and Mn+ animals (120 ± 40%). Baseline tension (~2000 mg) and precontraction tension developed in response to 10^{-7} NE (~788 mg) were similar before adding L-NMMA. The degree of NOS inhibition was verified in each vessel by adding 10^{-4} *M* ACh and observing <10% vasorelaxation.

Difluoromethylornithine administration after NE precontraction relaxed vessels from Mn– animals (41 ± 8%) and Mn+ animals (44 ± 6%) to the same extent. Further relaxation produced by ACh (10^{-8}–10^{-4} M) was not different between vessels from Mn– rats (10^{-4} *M* ACh = 68 ± 11%) and Mn+ rats (10^{-4} *M* ACh = 71 ± 10%). Baseline tension (~1996 mg) and precontraction tension developed in response to 10^{-7} NE (~718 mg) before adding DFMO were similar between groups.





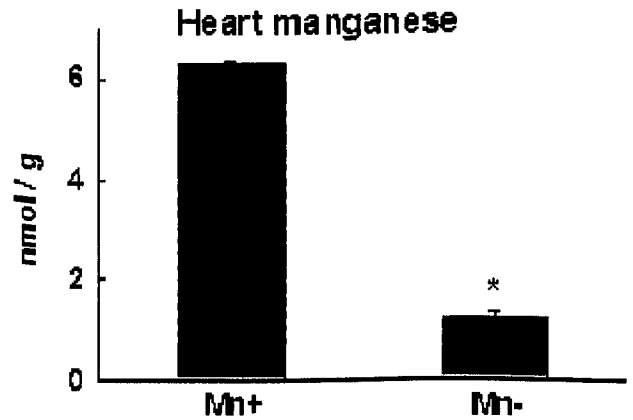
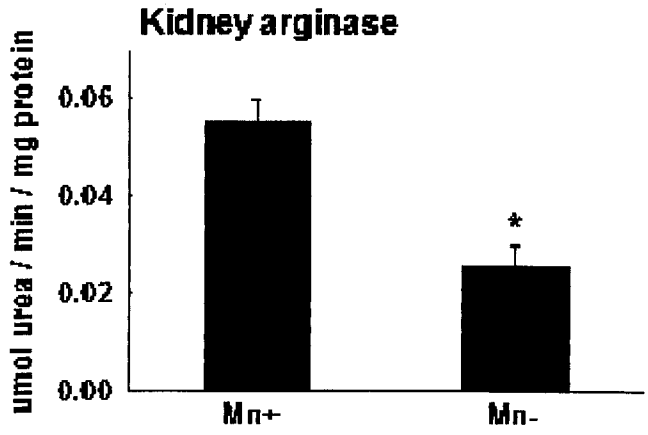


Figure 1. Liver, kidney, and heart manganese (Mn) concentrations from rats fed a manganese-sufficient (Mn+) or manganese-deficient (Mn–) diet (n = 6 per group). Values are mean ± SE. * $P \leq 0.05$ versus Mn+ animals.



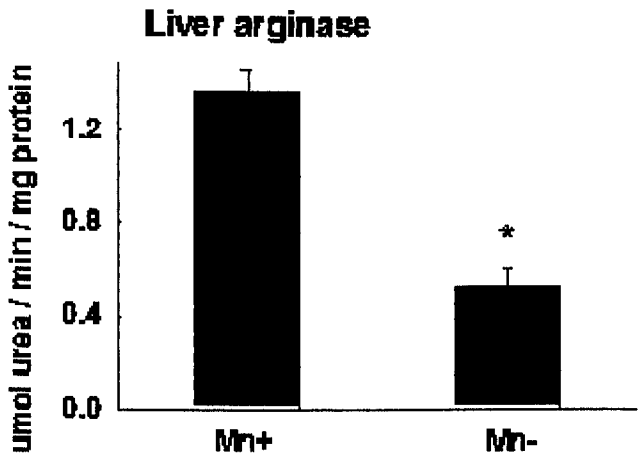


Figure 2. Kidney and liver arginase activities from rats fed a manganese (Mn)-sufficient (Mn+) or Mn-deficient (Mn–) diet (n = 12 per group). Values are mean ± SE. * $P \leq 0.05$ versus Mn+ animals.

Acetylcholine- and SNP-evoked vasorelaxation of coronary arteries were similar regardless of whether KCl (Fig. 4, Panels A and B) or ET-1 (Fig. 5, Panels A and B) was used as the precontractile agent. Baseline tension (~280 mg) and KCl-induced tension development (~153 mg) were similar in vessels from Mn– and Mn+ animals before adding ACh or SNP. Baseline tension (~178 mg) and ET-1–induced tension development (~214 mg) were similar in vessels from Mn– and Mn+ animals before adding ACh or SNP.

The L-NMMA–induced tension development was not different in coronary arteries from Mn– rats (30 ± 18%) versus Mn+ rats (16 ± 5%). Baseline tension (~192 mg) and ET-1–induced tension development (~136 mg) were similar in vessels from Mn– and Mn+ animals before adding L-NMMA. The degree of NOS inhibition was verified in each vessel by adding 10^{-4} M ACh and observing <10% vasorelaxation. The DFMO *per se* relaxed vessels from Mn– (22 ± 6%) and Mn+ (15 ± 5%) animals to the same extent. Further relaxation produced by ACh (10^{-8}–10^{-4} M) was not different between groups. For example,

Table 1. Tissue MnSOD and CuZnSOD Activities for Mn+ and Mn− Rats[a]

	MnSOD[b]			CuZnSOD[b]		
	Liver	Kidney	Heart	Liver	Kidney	Heart
Mn+	3.49 ± 0.13	4.13 ± 0.20	7.08 ± 0.30	12.12 ± 1.06	7.21 ± 0.41	4.14 ± 0.47
Mn−	2.48 ± 0.09*	2.47 ± 0.08*	4.22 ± 0.26*	9.82 ± 0.52	7.84 ± 0.40	5.05 ± 0.56

[a] MnSOD, manganese superoxide dismutase; CuZnSOD, copper and zinc superoxide dismutase; Mn+, manganese sufficient; Mn−, manganese deficient.
[b] MnSOD and CuZnSOD activities are expressed in U/mg protein (values are mean ± SE; $n = 12$ per group).
* $P < 0.05$ versus Mn+.

maximal relaxation produced by 10^{-4} *M* ACh was 66 ± 10% and 69 ± 11% for Mn− and Mn+ animals, respectively. Baseline tension (~195 mg) and ET-1–induced precontraction (~114 mg) before adding DFMO were similar between groups. Individual results from L-NMMA, DFMO, and DFMO + ACh experiments in KCl-precontracted vessels are not shown, but results were similar to those obtained in ET-1–precontracted vessels.

Amino Acid and Nitrate + Nitrite Analyses. Plasma arginine (μmol/L plasma) was higher ($P \leq 0.05$) in Mn− animals (116 ± 6) versus Mn+ animals (91 ± 8). Plasma citrulline and ornithine (μmol/L plasma), respectively, were similar in the Mn− rats (56 ± 4 and 39 ± 3) and Mn+ rats (51 ± 4 and 37 ± 2). Plasma nitrate + nitrite (μmol/L) were similar between Mn− rats (11 ± 1) and Mn+ rats (10 ± 1). Urine nitrate + nitrite (μmol/mmol creatinine) were similar in Mn− animals (29 ± 6) versus Mn+ animals (35 ± 12).

Immunohistochemical Analyses. Staining for eNOS (Fig. 6, Panel A) was similar in aorta from Mn− animals (3.43 ± 0.44) and Mn+ animals (3.36 ± 0.53) as determined by scoring on a scale of 1 to 5 (1 = lowest staining intensity and 5 = highest staining intensity). In contrast, staining for nitrotyrosine was more intense ($P \leq 0.05$) in aortic sections from Mn− rats (3.71 ± 0.49) versus Mn+ rats (2.43 ± 0.37; Fig. 6, Panel B).

Table 2. Vessel Characteristics[a]

	Mn+	Mn−
Aorta		
i.d., baseline (μm)	746 ± 20	694 ± 17
i.d., 2 g tension (μm)	1409 ± 16	1378 ± 20
Vessel length (μm)	1764 ± 35	1704 ± 53
Coronary (KCl precontraction)		
i.d., baseline (μm)	114 ± 9	122 ± 7
i.d., L_{max} (μm)	273 ± 15	271 ± 14
Vessel length (μm)	1417 ± 68	1333 ± 60
Coronary (ET-1 precontraction)		
i.d., baseline (μm)	119 ± 4	118 ± 4
i.d., L_{max} (μm)	215 ± 8	215 ± 8
Vessel length (μm)	1346 ± 68	1504 ± 74

[a] Mn+, manganese sufficient; Mn−, manganese deficient; i.d., inside diameter; KCl, potassium chloride; ET-1, endothelin-1; L_{max}, the artery diameter that evokes the greatest tension development. Values are mean ± SE.

Discussion

The primary purpose of the present study is to determine whether lowering arginase activity *via* dietary Mn deficiency could enhance endothelium-dependent vasorelaxation. We reasoned that by attenuating arginase

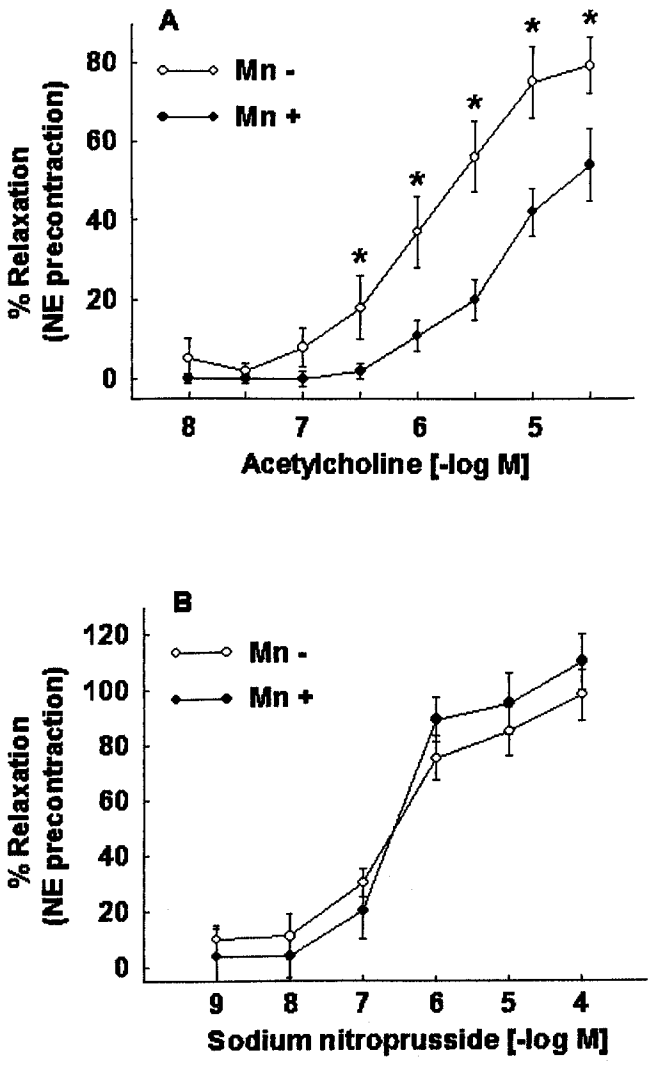
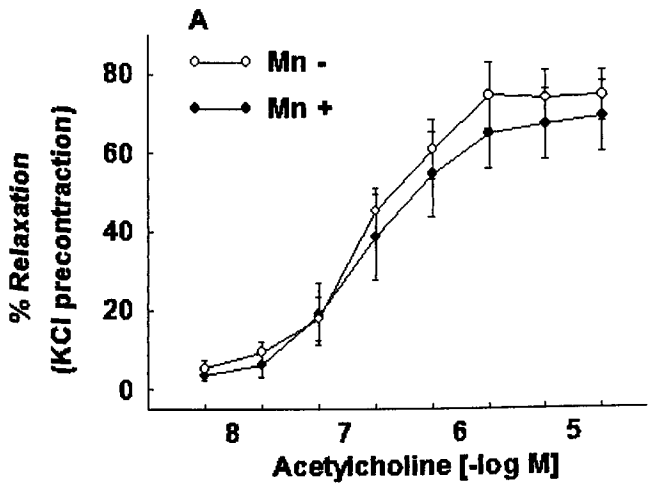


Figure 3. (Panel A) Acetylcholine (ACh)-evoked and (Panel B) sodium nitroprusside–evoked vasorelaxation in aortic segments from rats fed a manganese (Mn)-sufficient (Mn+) or manganese-deficient (Mn−) diet. Norepinephrine (NE; 10^{-7} *M*). Data represent 12 animals per group and 2 vessel segments per animal. Values are mean ± SE. * $P \leq 0.05$ versus Mn+ animals.



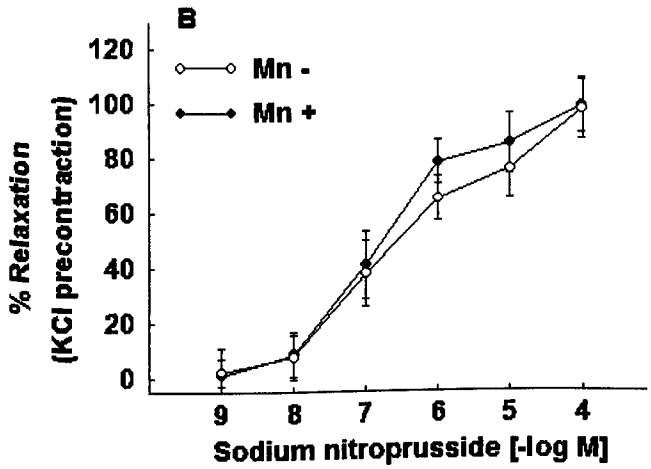
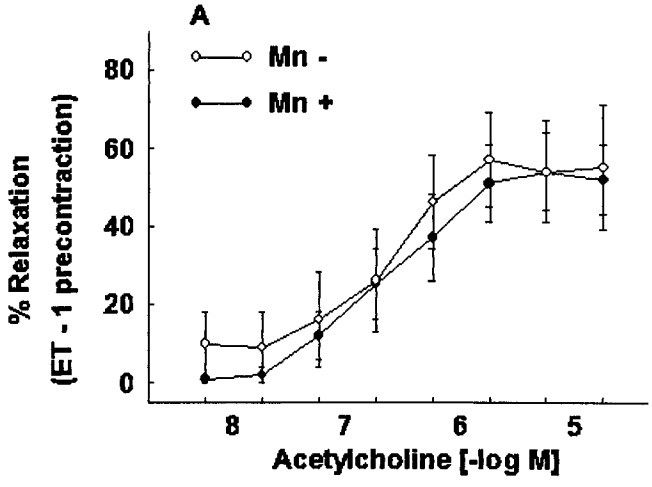


Figure 4. (Panel A) Acetylcholine (ACh)-evoked and (Panel B) sodium nitroprusside–evoked vasorelaxation in coronary arteries from rats fed a manganese-sufficient (Mn+) or manganese-deficient (Mn–) diet. Potassium chloride (KCl; 45 m*M*). Data represent 6 animals per group and 2 vessel segments per animal. Values are mean ± SE.



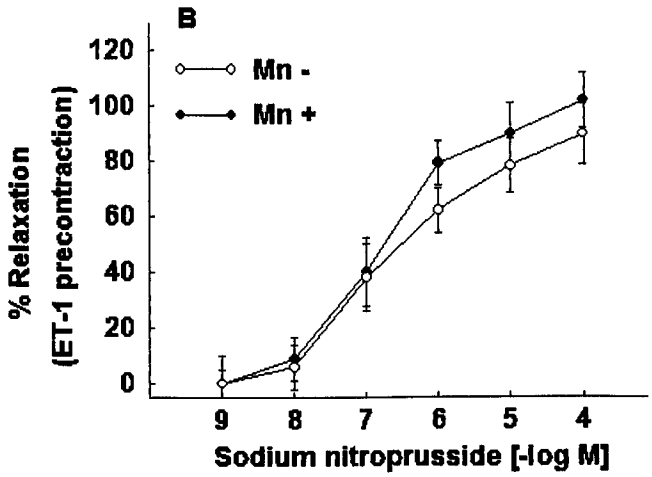


Figure 5. (Panel A) Acetylcholine (ACh)-evoked and (Panel B) sodium nitroprusside–evoked vasorelaxation in coronary arteries from rats fed a manganese-sufficient (Mn+) or manganese-deficient (Mn–) diet. Endothelin-1 (ET-1; ~3×10^{-8} *M*). Data represent 6 animals per group and 2 vessel segments per animal. Values are mean ± SE.

activity, more substrate (i.e., L-arginine) would be available for NOS, subsequent production of NO, and resultant vasorelaxation. Results from aortic segments indicate that ACh-evoked vasorelaxation was greater, whereas SNP-induced relaxation was unchanged, in Mn– versus Mn+ animals. These findings suggest that endothelium-dependent relaxation is improved, whereas nitric oxide/cGMP-dependent intracellular signaling pathways within vascular smooth muscle are unchanged, in Mn– versus Mn+ rats. Our immunohistochemical and biochemical results indicate that relative to Mn+ rats, Mn– rats have lower liver and kidney arginase activity (the enzyme that competes with eNOS for L-arginine), more plasma L-arginine (the substrate required for NO production), and similar expression of aortic eNOS (the enzyme that catalyzes the conversion of L-arginine to NO + citrulline). On the basis of our data, we propose that when ACh binds to muscarinic receptors and releases calcium that subsequently activates eNOS, more substrate (i.e., L-arginine) is available to form NO and L-citrulline than ornithine and urea because arginase activity is reduced. This explanation is based on the premise that NO release evoked by muscarinic receptor stimulation is primarily responsible for vasorelaxation in rat aortae. Evidence from our laboratory supports this. For example, preliminary studies for this investigation indicate that atropine (10^{-6} mol/L) abolishes ACh-evoked relaxation. Further, ACh-evoked relaxation was attenuated by ≥90% in the presence of L-NMMA

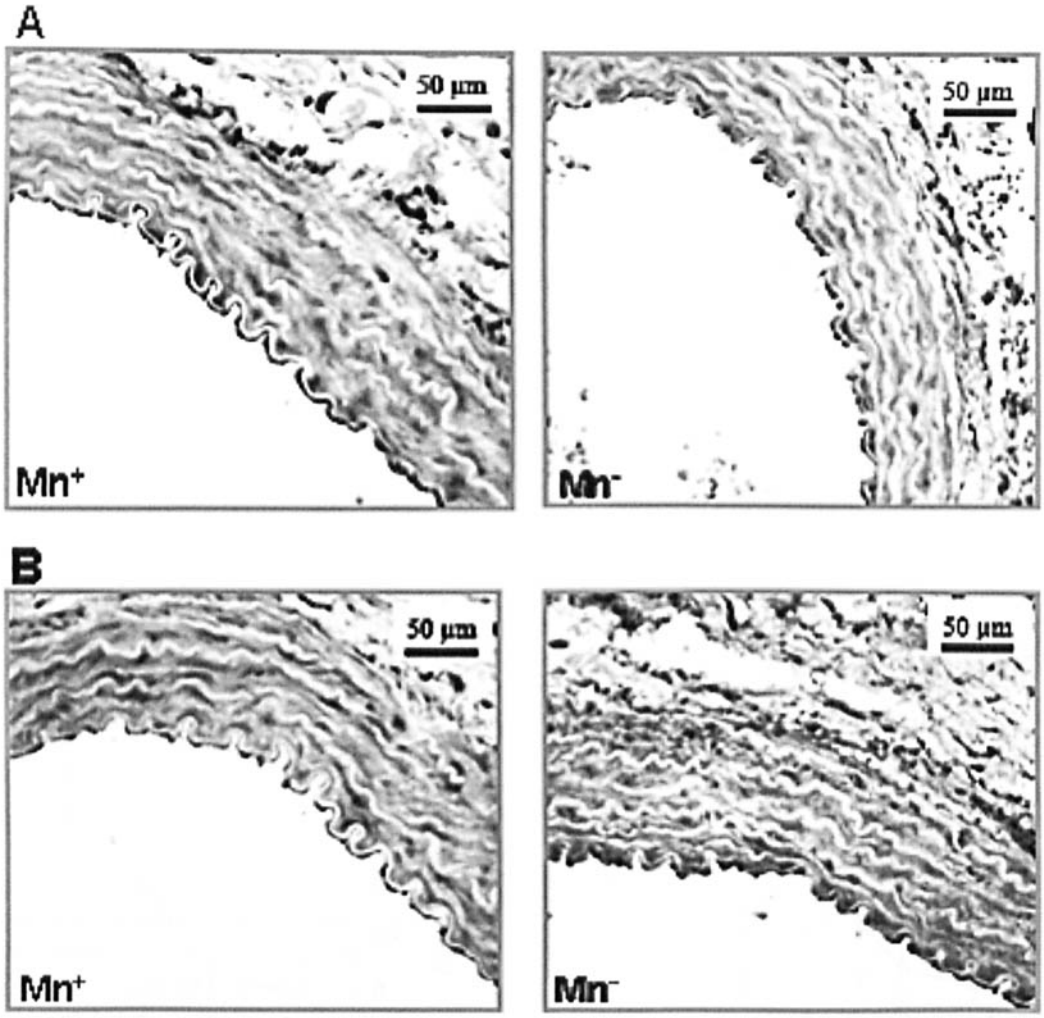


Figure 6. Transverse sections of aorta (×40) from rats fed a manganese-sufficient (Mn+; $n = 6$) or manganese-deficient (Mn–; $n = 6$) diet. (Panel A) Immunohistochemistry results indicate similar staining between groups for endothelial cell nitric oxide synthase (brown stain predominantly localized in the luminal endothelial cells. (Panel B) In contrast, a greater number of cells stained positively for nitrotyrosine (brown stain seen throughout the lumen) from aorta of Mn– versus Mn+ animals ($P \leq 0.05$).

(10^{-3} mol/L). Taken together, these findings support our hypothesis that Mn deficiency can reduce arginase activity to an extent that endothelium-dependent vasorelaxation of aortic segments is improved.

Although ACh-evoked vasorelaxation is useful for the pharmacologic assessment of stimulated NO production and its functional consequences (i.e., vasorelaxation), inhibiting NOS using L-NMMA is a method whereby basal NO production can be estimated (33). We observed that L-NMMA–induced vasocontraction was not different between the two groups, suggesting similar basal NO production in aortic segments from Mn– and Mn+ rats. These results are consistent with our findings that neither eNOS expression nor plasma/urine nitrate + nitrite were different among animals from each group. Further, if basal NO was elevated to an extent that influenced total peripheral resistance, we likely would have observed lower arterial blood pressures in Mn– versus Mn+ animals. Collectively, our data suggest that stimulated, but not basal, NO production is greater in the presence of reduced arginase activity evoked by consuming a Mn-deficient diet.

Vascular responses to inhibiting arginase activity acutely using DFMO were also evaluated in aortic segments. In the first part of this protocol, we administered a dose of DFMO previously shown to suppress arginase activity without affecting NOS activity (4). We reasoned that DFMO would increase L-arginine availability for eNOS and result in vasorelaxation when administered to precontracted vessels. Because lower arginase activity was observed in Mn– animals, we hypothesized that DFMO-induced vasorelaxation would be greater in Mn+ versus Mn– rats. This hypothesis was not supported. Instead, arterial vasorelaxation to DFMO *per se* was similar between groups. At least two potential explanations exist for this finding: (i) sufficient arginase activity may have been available, even in Mn– animals, such that DFMO-induced inhibition of this enzyme

was similar between groups, and (ii) DFMO-induced arginase inhibition may have increased L-arginine availability for enzymes other than NO synthase. Although we cannot definitively conclude the existence of either of these possibilities, they are not unreasonable to consider.

In the second portion of the DFMO protocol, we administered cumulative doses of ACh after the initial relaxation response to DFMO had stabilized. We reasoned that if improved vasorelaxation observed in Mn– rats (Fig. 3, Panel A) resulted from lower arginase activity and redirection of L-arginine through the eNOS pathway (our original hypothesis), then acute arginase inhibition (i.e., by DFMO) should improve ACh-evoked vasorelaxation, even in Mn+ animals. Our results in the thoracic aorta support this contention. Specifically, maximal ACh-evoked relaxation in vessels from Mn+ animals increased ($P \leq 0.05$) from 54 ± 9% in the absence of DFMO (Fig. 3, Panel A) to 71 ± 10% in the presence of DFMO. Taken together, our findings indicate that ACh-stimulated vasorelaxation of the thoracic aorta is greater after both chronic (i.e., *via* dietary Mn deficiency) and acute (i.e., *via* DFMO) inhibition of arginase activity.

Protocols similar to those described for the aorta also were performed on small coronary arteries. Vessels of this size are important because they are largely responsible for regulating myocardial blood flow (34, 35). Consistent with the only other study to examine the effects of acute arginase inhibition on coronary vascular reactivity (4), we showed that DFMO-induced vasorelaxation is potentiated by stimulated (i.e., ACh-evoked) NO release. Furthermore, we compared endothelium-dependent coronary vasorelaxation among animals wherein arginase activity was lowered *via* long-term dietary Mn deficiency (i.e., Mn– rats) to those that consumed purified diet (i.e., Mn+ rats). In contrast to our findings in the aorta, coronary vasorelaxation was similar between vessels from the Mn+ and Mn– groups. Although these results do not support our original hypotheses, findings from other analyses we performed may help in their interpretation. For instance, consistent with previous studies from our laboratory (10) and others (36, 37), we observed lower MnSOD activity in heart tissue from Mn– versus Mn+ animals. Lower MnSOD activity could compromise the antioxidant environment, resulting in elevated oxidant load (i.e., superoxide anion). Indirect evidence for this in our study is that nitrotyrosine staining was more pronounced in Mn– versus Mn+ animals. Nitrotyrosine is an estimate of peroxynitrite formed from the reaction between NO and superoxide anion (38). Because our estimates of eNOS activity (i.e., immunostaining, tension evoked by L-NMMA, plasma/urine nitrate + nitrite levels) were similar between groups, and MnSOD activity was lower in Mn– rats, elevated nitrotyrosine in Mn– animals likely resulted from an increased concentration of superoxide anion. Because small coronary arteries are particularly susceptible to damage evoked by superoxide anion (39, 40), the potential for greater coronary vasorelaxation in Mn– animals may have been masked by increased oxidant load. Although this explanation is reasonable, it cannot be made with certainty because MnSOD was measured in heart tissue rather than directly in vascular tissue.

Another possible explanation for our finding that endothelium-dependent vasorelaxation was similar in coronary arteries regardless of whether a Mn– or Mn+ diet was consumed is that the procedures we used to measure coronary reactivity may have been insensitive to subtle differences potentially produced by Mn deficiency. Several methods exist to evaluate arterial function, and each has positive and negative aspects. For this study, we used a wire-type myograph that allows direct determination of vessel wall force in the absence of influences from neural, humoral, metabolic, and mechanical sources (41, 42). Another common method is to quantify vasodilation and vasocontraction using video microscopy of pressurized and cannulated vessels (4). Although differences may have been observed between groups using techniques other than those we employed, it must be noted that we have detected functional changes in small coronary arteries due to other nutritional interventions (17), myocardial ischemia (16, 18, 23), pharmacologic inhibition of receptors (16), and physical exercise (18, 23) using techniques identical to those described in the present study.

Concern may be raised that arginase activity was not measured directly in the vascular tissue. These assays were attempted in heart, aorta, and coronary tissue, but results were considered unreliable for reasons explained in Materials and Methods. In any case, quantification of arginase I in the liver and arginase II in the kidney verified that Mn deficiency lowered arginase activity in our study.

This is the first study to demonstrate that consuming a Mn-deficient diet improves endothelium-dependent vasorelaxation of rat aortic segments. Our immunohistochemical and biochemical results indicate that, relative to Mn+ rats, Mn– rats have lower arginase activity, more plasma L-arginine, and similar eNOS expression. Therapeutic potential may exist for lowering arginase activity *via* Mn deficiency during pathophysiologic conditions associated with compromised vascular function (e.g., diabetes, hypertension). One would only envision using such an approach over a short time given the essential nature of Mn. For example, caution would need to be exercised to ensure that the positive vascular effects of Mn deficiency are not outweighed by increased tissue oxidative damage resulting from a compromised antioxidant environment and elevated oxidant load (43). Clearly, the consequences of a dietary Mn deficiency and/or low Mn status on nitric oxide–mediated pathways warrant further investigation.

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