8-2005

The Effect of Dietary Manganese on Arterial Functional Properties

Anastasia Z. Kalea

THE EFFECT OF DIETARY MANGANESE ON ARTERIAL FUNCTIONAL PROPERTIES

By

Anastasia Z. Kalea

B.Sc. Harokopio University of Athens, Greece, 2000

A THESIS

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

(in Food and Nutrition Sciences)

The Graduate School

The University of Maine

August, 2005

Advisory Committee:

Dorothy J. Klimis-Zacas, Professor of Clinical Nutrition, Advisor

Rodney J. Bushway, Professor of Food Science and Human Nutrition

Patrick D. Harris, Professor of Physiology and Biophysics

Linda Kling, Associate Professor of Marine Sciences

Dale A. Schuschke, Associate Professor of Physiology and Biophysics

THE EFFECT OF DIETARY MANGANESE ON ARTERIAL FUNCTIONAL PROPERTIES

By Anastasia Z. Kalea

Thesis Advisor: Dr. Dorothy J. Klimis-Zacas

An Abstract of the Thesis Presented
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy
(in Food and Nutrition Sciences)
August, 2005

Dietary manganese affects the structure and integrity of blood vessels, as well as vessel predisposition to endothelial dysfunction and cardiovascular disease. In this thesis, we studied the role of manganese on the functional properties of rat aorta as defined by the endothelial and vascular smooth muscle cell pathways for adrenergic-mediated vasoconstriction and cholinergic-mediated vasodilation.

Weanling Sprague-Dawley rats were fed a manganese deficient (MnD), adequate (MnA-control group) or supplemented (MnS) diet (<1, 10-15 and 45-50ppm Mn respectively). After 14 weeks on the diet the aorta was excised and four aortic rings of three mm length were prepared from each animal. Alterations in vasoconstriction among diet groups were detected by dose-response curves to the α_1-adrenergic agonist L-Phenylephrine in endothelium-intact and endothelium-disrupted rings. Alterations in endothelium-dependent vasodilation among diet groups were determined by dose-response curves to Acetylcholine. We studied the mechanism by which dietary manganese affects two different endothelium-dependent vasodilation pathways: the L-

arginine/nitric oxide (NO) and the cyclooxygenase (COX) pathways. Inhibition of the enzymes for NO synthesis (NOS) with L-NMMA, and of prostanoids (COX I and II) with Mefenamic acid, determination of NOS expression, and *in vitro* addition of L-Arginine (substrate for NO formation) to vessel rings revealed the effect of manganese on the regulation of endothelium-mediated vasodilation and vasoconstriction. Dose-response curves to sodium nitroprusside provided data for the dietary effect on endothelium-independent vasodilation.

Supplementary dietary manganese increased adrenoreceptor-mediated vascular smooth muscle contraction, which was significantly reduced in the presence of functional endothelium. Absence of dietary manganese increased endothelial cell sensitivity to the α_1-adrenergic vasoconstrictor agent. Manganese had a small effect on the cGMP-pathway for dilation of vascular smooth muscle but affected vasodilation primarily through an endothelium-mediated pathway, probably by preserving NO bioavailability. Inhibition of vasodilation in Mn deficiency appears to occur through an endothelium-derived vasoconstrictor, possibly thromboxane with a concomitant decrease in the synthesis of endothelium-mediated vasodilator prostanoids. Our results demonstrate that dietary manganese influences the contractile machinery of vascular smooth muscle cells and regulates the bioactivity of endothelium-mediated vasodilators to affect agonist-induced signaling pathways that participate in the regulation of vasomotor tone. This suggests possibilities for dietary intervention in blood pressure regulation.

DEDICATION

This is dedicated to Dad, Mom, my sister Dora and my Grandparents Markos, Anastasia, Athanasios and Theodora, for their love, caring, continuous support and encouragement.

ACKNOWLEDGMENTS

I would like to thank my thesis advisor Dr. Dorothy Klimis-Zacas, for her guidance and support during the course of my studies and dissertation research. Her expertise and generosity of time helped me to make this thesis possible. I was motivated by her zeal, dedication and commitment to research in order to persist in solving my questions. Her patience, understanding and kindness will always inspire my admiration of her as a scientist and as a person.

A special thank you to my committee members from the University of Louisville, Dr. Patrick Harris and Dr. Dale Schuschke for their continuous guidance during all the different steps of my study, for their careful review of this dissertation and for the opportunities they offered me all these years to be trained and explore new, highly sophisticated and fascinating research methodologies. I am very grateful to Dr. Linda Kling for her encouragement and her enthusiasm on my work and results. A very special thanks to Dr. Rod Bushway for his willingness to assist me in any possible way in my thesis project but also for his time and effort to find a solution in any problems I had to face as a graduate student during the last four years.

I am especially grateful to all the friends that trained me at the University of Louisville: Touichi Kawabe, M.D. who introduced me in the microsurgical techniques and the pharmacological/ physiological protocols and methodologies, as well as Dr. Nina Tsakadze and Dr. Leila Gobejishvili for being always available to answer my questions

and to guide me. Additionally, my four-month training on glycosaminoglycan analyses research methods at the University of Patras, Greece offered me a unique experience as a researcher but also strengthened my knowledge and expanded my skills on biochemical techniques. I am extremely grateful to have worked with Dr. Fotini Lamari, whose experience in the lab and zeal for her work greatly inspired me. A special thank you to Dr. Karamanos, Dr. Theocharis, Dr. Roussidis, Yiannis, Kostas, Maria, Athanasia and Olga for being so generous to teach me new techniques and share with me their lab experience.

I would like to express my special appreciation to Kateryna Clark, since without her invaluable assistance in the lab and her friendship it would have been impossible to complete my experiments. A special thank you to my friends and colleagues Sandra Brussee, Sara Aldaous and Natasha D'Souza for their kindness, generosity and continuous support. I also want to thank the Director of the Graduate School, Dr. Scott Delcourt and all the faculty members and staff of the Dept. of Food Science and Human Nutrition, Dept. of Biochemistry, Dept. of Plant and Soil and Environmental Sciences, Dept. of Marine Sciences of the University of Maine, especially Brenda Kennedy-Wade, Brian Perkins, Dulip Larkshman, Louis Morin, Lauren Crawford, Judy Polyot, Dr. Gundersen and Dr. Elskus.

Being away from my family and my country during the last four and a half years was a new experience for me. However, the support of the Greek community of Bangor and of the community of St. George Greek Orthodox Church made me feel that I had a new

family here. Special thanks to Miltiades Zacas, whose kindness, invaluable advice and encouraging words will always inspire my admiration of him. Much appreciation to Fr. Adam Metropoulos and Presvytera Lisa, Yiannis, Heleni, Lambros, Emily, Kyriacos, Vasia, Konstantinos, Alexis, Elizabeth, Polly, Kiki, Michalis, Rena, Dimitris, Jorgos and their family. Special thanks to my friends Sotiris and Charalampos for always being there. I am very fortunate to have met dear friends in the University of Maine, who made all these years that I spent here very pleasant ones. It was very important for me to have Riccardo, Vishesh, Anand, Sanjeev, Chie, Luiz and Eduardo to share my successes and joy as well as my difficult times. Many special thanks to all my family, friends and colleagues from Greece, Maria, Rania, Irene, Constandia, Giota, Makis, Zoe, Christos, Nafsika, my cousin Dorina that kept e-mailing and calling so frequently in order to support me in any difficult decision and to Fr. Georgios for his care and priceless guidance. I am especially grateful to my friend Maria Amenda, who has always been there with her love, support, wishes and thoughts.

A very special thank you goes to my parents, my sister Dora and my grandparents for their love, support and encouragement during the course of my studies. I feel extremely blessed to have such a loving family; even though they were in Greece it felt that they were always next to me and I owe them everything that I have accomplished.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

Manganese and Arterial Functional Properties: An Overview of the Literature

1.1. Manganese as an Essential Trace Element

Manganese (Mn) belongs to the group of fourteen essential trace elements and is named after the Greek word for "magic", which represents the wide range of metabolic functions and diverse pathologies that can result from either Mn deficiency or toxicity [Cotzias, 1958]. Manganese is present in most mammalian tissue in concentrations ranging from 0.3 to 2.9 μg Mn/g of wet tissue weight and is considered to be among the least toxic of trace elements when administered orally [Dorman, 2001]. Manganese enters the body through dietary sources and through combustion of gasoline that might release air-borne submicron particles of Mn_3O_4 [Barceloux, 1999]. Food is the main source of Mn intake, which varies from 2-9 mg daily [Greger, 1999]. Manganese is absorbed from the diet in divalent and tetravalent states [Barceloux, 1999]. The bioavailability of Mn depends on the physiological mechanisms that regulate Mn absorption, liver metabolism, biliary and pancreatic excretion and this Mn bioavailability is influenced by the parallel intake of other nutrients [Britton & Cotzias, 1966][Davis et al., 1993][Finley & Davis, 1999][Malecki et al., 1996] [Papavassiliou et al., 1966][Kuratko, 1997][Johnson, 1990].

Food is the most important source of Mn for the general population. Daily intake varies from 2-9 mg Mn depending on dietary habits, the consumption of high Mn-containing foods and the Mn concentration of the soil that subsequently affects the

concentration of Mn in water and food [Barceloux, 1999][Patterson et al., 1984]. Rich dietary sources of Mn are nuts, grains, tea, legumes, fruits and vegetables. Poultry, meat, refined foods and dairy products comprise poor sources of the trace element [Gropper et al., 2004].

Manganese deficiency results in skeletal abnormalities in many animal species [Wolinsky et al., 1994]. Shortened and thickened bones with swollen and enlarged joints, chondrodystrophy, disturbed synthesis of organic matrix of cartilage and bone are some of the physical abnormalities [Tal et al., 1965][Leach, 1971]. In Mn deficiency development of abnormal otoliths in the inner ear can cause ataxia and inability to control body-righting reactions [Freeland-Graves et al., 1994], as well as abnormalities in function and ultrastructure of mitochondria [Liu et al., 2002]. As the site of oxidative phosphorylation, mitochondria are a significant source of potentially hazardous reactive oxygen species and free radicals. Thus, deficiency of Mn might lead to superoxide radical poisoning due to the loss of manganese superoxide dismutase (Mn-SOD) activity, a Mn-dependent antioxidant enzyme of mitochondrial origin [Liu et al., 2002][Yan et al., 1998][Yan et al., 2001]. Clinical symptoms of experimental Mn deficiency have been described in two human studies. Doisey reported development of scaly transient dermatitis, hypocholesterolemia, depressed vitamin K-dependent clotting factors and reddening of hair with an accidental low Mn intake of 0.34 mg/ day in one human [Doisey, 1972]. In a second study, an experimental semipurified diet of 0.11 mg/day was consumed by seven men between the ages of 19 and 22 years for 39 days, and they developed a reduction in total and high density lipoprotein serum cholesterol, fine scaling, and *miliaria crystallina* which was defined as a type of erythematous rash

[Friedman et al, 1988]. In contrast, there have been no reports of gross Mn deficiencies in free-living populations, probably due to the abundance of Mn in the diet and the very low requirements for Mn [Underwood, 1981].

Methodological difficulties such as the lack of correlation between serum Mn and tissue levels, increase the difficulty in assessing and documenting Mn deficiency [Matsuda et al., 1989]. However, several population groups have been characterized with suboptimal levels of Mn. These include children with metabolic syndromes such as phenylketonuria, maple syrup disease, galactosemia and methylmalonic acidemia; children and adults with epilepsy; patients with exocrine pancreatic insufficiency, active rheumatoid arthritis, or hydralazine syndrome [Freeland-Graves, 1988][Dupont & Tanaka, 1985]. Pennington et al. [Pennington et al., 1986] have reported that mean Mn intakes for adolescent girls, adult and elderly women are considerably lower than the level presently set by the Estimated Safe and Adequate Daily Diet Intakes. Intake of Mn may also be compromised in the U.S. population since routine use of supplements, such as calcium, as well as high intakes of processed foods and refined carbohydrates might reduce absorption or retention of Mn [Temple, 1983].

Manganese toxicity can be a result of acute or chronic exposure to the trace element due to occupational inhalation of Mn-containing particles or fumes in Mn mines and other industrial plants. The symptoms of Mn toxicity resemble the ones in Parkinson's disease and are associated with a decrease in dopamine levels in the dopaminergic nigrostriatal pathways [Mena, 1970][Keen, 1984]. To date, there are no reports of Mn toxicity in humans associated with food or water consumption.

1.2. The Biochemical Role of Manganese

Manganese participates as a cofactor in the structure of several enzymes and modulates important signal transduction pathways (Table 1.1)[Wedler, 1994]. Manganese increases the accumulation of second messengers such as cyclic AMP (cAMP) and cyclic GMP (cGMP) by activating adenylate cyclase (AC) and guanylate cyclase (GC) respectively, which are significant diffusible effector molecules that activate proteins for further cell-signaling [Korc, 1993][Keen et al., 1984]. Manganese might modulate *in vitro* cell-surface receptor binding and adhesion by modifying the requirement for specific adhesion proteins (Table 1.2.) [Edwards et al., 1988]. The effects of Mn on cytoplasmic Ca^{+2} levels might have an indirect effect on cellular function. Increases in intracellular Ca^{+2} levels might occur with the entrance of Ca^{+2} directly from extracellular sites by transmembrane diffusion, voltage dependent channels or agonist dependent channels [Gropper et al., 2005]. Manganese can modulate Ca^{+2} homeostasis by blocking Ca^{+2} influx, by enhancing Ca^{+2} efflux and by increasing Ca^{+2} flux through the mitochondrial uniporter [Korc, 1993][Kwan et al., 2003]. In addition to its role as a Ca^{+2} entry blocker, Mn has been shown to enhance the autoxidation of catecholamines to attenuate the contractile response to norepinephrine *in vitro* [Kappus & Schenkman, 1979].

Table 1.1. Manganese-dependent enzyme systems [adopted in part from Wedler, 1994].

Category	Function
Oxidoreductases	
Superoxide dismutase	$O_2^{-\cdot}$ acceptor
Catalase	H_2O_2 acceptor
Peroxidase	H_2O_2 acceptor
Transferases	
Galactosyl-	Glycosyl groups
Glucosyl-, glycosyl-	Glycosyl groups
Glucuronosyl-	Glycosyl groups
Protein kinases	P-containing –OH acceptor
(Tyr, Ser) protein kinases	P-containing –OH acceptor
P-inositol(4-P)kinase	P-containing –OH acceptor
Sulfo-groups	S-containing
Hydrolases	
Protein phosphatases	P-monoesters
P-inositol phosphatase	P-monoesters
Cyclic nucleotide phophodiesterase	P-diesters
Metalloproteases	Peptide bonds
Arginase	Amidines
Lyases	
PEP carboxykinase	C-C bonds
Adenylate cyclase	P-O bonds
Guanylate cyclase	P-O bonds
Ligases	
Glutamine synthetase	C-N bonds
Pyruvate carboxylase	C-C bonds
AA-tRNA synthetases	C-O bonds

Manganese is an essential component of several metalloproteins such as pyruvate carboxylase and glutamine synthetase. The role of Mn in glucose metabolism has not yet been clearly established even though it appears to be the preferred divalent cation for activation of the protein tyrosine kinase activity of the insulin receptor [Rosen & Lebwohl, 1988]. Manganese is also a co-factor for enzyme systems such as xanthine oxidase, calmodulin-dependent protein phosphatase, hexokinase, arginase and superoxide dismutase [Wedler, 1994][Korc, 1993][Weisiger et al., 1973]. MnSOD plays a dominant role in protecting cells from oxidative stress by catalyzing the reaction of superoxide radical to hydrogen peroxide, a less reactive oxygen species. MnSOD requires Mn as a transition metal co-factor. Thus, MnSOD activity might be controlled through availability of Mn. Hence, the antioxidant effect of Mn can be attributed to a large extent to the role of Mn as a cofactor of MnSOD, whose activity is directly related to the nutritional status of Mn [Fernandes et al., 1988][Malecki & Greger, 1996].

Special interest has been focused lately on arginase, a regulatory enzyme that is allosterically activated by Mn and has the potential of regulating the availability of L-arginine for nitric oxide (NO) synthesis, an endothelium-derived vasodilator. Rat aortic endothelial cells express two types of arginases (I and II) with different subcellular localization. Altered arginase activity could limit the availability of L-arginine for NO synthesis in intact cells [Wu & Morris, 1998]. Severe Mn deficiency reduces arginase activity, which implicates that Mn might affect NO production and/or bioavailability.

The diffusion of NO from the endothelial cell to the vascular smooth muscle cell leads to activation of soluble guanylate cyclase (sGC), a metalloenzyme that prefers Mn^{+2} over Mg^{+2} as a cofactor to support its catalytic activity [Lucas et al., 2000]. Nitric oxide binds

directly to the heme component of sGC to form a ferrous-nitrosyl-heme complex [Nighorn, 1999]. Cyclic GMP is formed from GTP, which induces smooth muscle relaxation by reducing intracellular Ca^{+2} and desensitizing the contractile apparatus to Ca^{+2}. The precise mechanism by which Mn^{2+} allosterically modulates guanylyl cyclase activity remains unclear.

The skeletal and structural abnormalities that are observed in Mn deficiency are due to the decreased activity or expression of Mn-dependent enzymes that participate in mucopolysaccharide synthesis. These enzymes include UDP-xylosyltransferase, glycosyltransferases, galactosyltransferases I and II and polymerase, which mediate the synthesis and sulfation pattern of chondroitin sulfate [Leach, 1969][McNatt et al., 1976][Yang & Klimis-Tavantzis, 1998][Yang & Klimis-Tavantzis, 1998b]. Manganese seems to affect not only the concentration but also the sulfation pattern and disaccharide composition of aortic heparan sulfate glycosaminoglycans [Kalea et al., 2004]. The sulfation of proteoglycan molecules might affect the clustering and thus the activation of cellular receptors [McDonnel et al., 2004].

Table 1.2. Receptor-Manganese Interactions [adopted in part from Wedler, 1994].

Receptors
Acetylcholine
Adrenergic
Cholinergic
Dopaminergic
Fibronectin
Insulin
Manose-6-P
Muscarinic
Opioid
Oxytocin
Platelet GPIIb/IIIa
Serotonin
Tachykinin
Vasopressin

1.3. Endothelial Function and Dysfunction

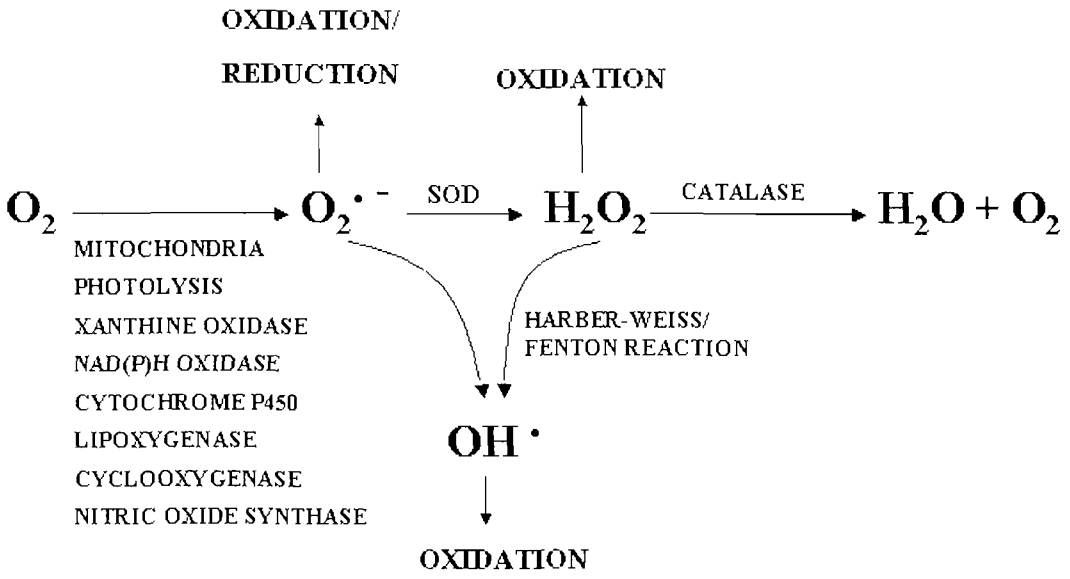
During the past two decades medical research has increasingly focused on the relationship between endothelial function and the different stages of various cardiovascular diseases. The endothelial cell layer is the largest endocrine organ in the body since the endothelium lines the vascular lumen throughout the cardiovascular system. The endothelium forms an interface between tissues and circulating blood, which provides oxygen, nutrients and hormones and removes carbon dioxide and other metabolic waste products [van Hinsbergh et al., 2002]. The diverse biological roles of endothelium include regulation of vasomotor tone, cellular permeability, intravascular homeostasis, platelet aggregation, monocyte adhesion, vascular smooth muscle growth and blood coagulation [Matsuoka, 2001].

Endothelial modulation of vascular tone is mediated primarily through the production and release of endothelium-derived vasoactive modulators that affect directly or indirectly the contractile machinery of the vascular smooth muscle [Lüscher & Vanhoutte, 1990]. These endothelial modulators are divided into two classes: the endothelium-derived relaxing factors (EDRFs) and the endothelium-derived contracting factors (EDCFs). Nitric oxide (NO), prostacyclin and endothelium-derived hyperpolarizing factor (EDHF) are major relaxing factors that cause dilation of vascular smooth muscle. Endothelin-1, thromboxane, prostaglandin H_2, catecholamines and angiotensin II are major contracting factors. However, a complete list of all EDRFs and EDCFs still remains unknown.

Endothelial dysfunction is a hallmark of atherosclerosis and is characterized by an imbalance between the EDRFs and EDCFs that precedes the early manifestations and angiographic detection of cardiovascular disease [Vogel, 1997]. Endothelial cells in the intimal layer of the blood vessel wall recognize the presence of vasoactive substances such as acetylcholine, adenosine di- and tri-phosphate, histamine, bradykinin and norepinephrine to generate signals that modify the contractile machinery of the smooth muscle cells in the media layer [Vanhoutte, 1981]. One major cause of endothelial dysfunction is oxidative stress, which results from abnormalities in the mitochondrial respiratory chain or in the coupling of oxidative phosphorylation [Matsuoka, 2001]. The superoxide anion is reduced to hydrogen peroxide via dismutation and transition metal-catalyzed reactions lead to the production of highly reactive hydroxyl radicals from hydrogen peroxide (Figure 1.1). Reactive oxygen species interact with endogenous vasoactive mediators and modulate vascular tone and the atherogenic process [Arnal et

al., 1999]. Superoxide reacts rapidly with nitric oxide to inactivate it. Oxidative stress also reduces the endogenous synthesis of endothelial nitric oxide synthase (eNOS or NOS III), the enzyme that catalyzes the formation of NO from L-arginine [Wang et al., 2000].

Figure 1.1. Generation of Reactive Oxygen Species.



The role of the endothelium as a protagonist in vascular homeostasis is evidenced by the fact that disease conditions such as essential hypertension, atherosclerosis and diabetes involve an impaired production or bioavailability of endothelium-derived mediators [Rubanyi, 1988][MnIntyre et al., 1997][De Vriese et al, 2000]. The exposure to coronary risk factors decreases the bioactivity to EDRFs and increases the release of EDCFs. Aging, prolonged exposure to shear stress and lifestyle-related risk factors such

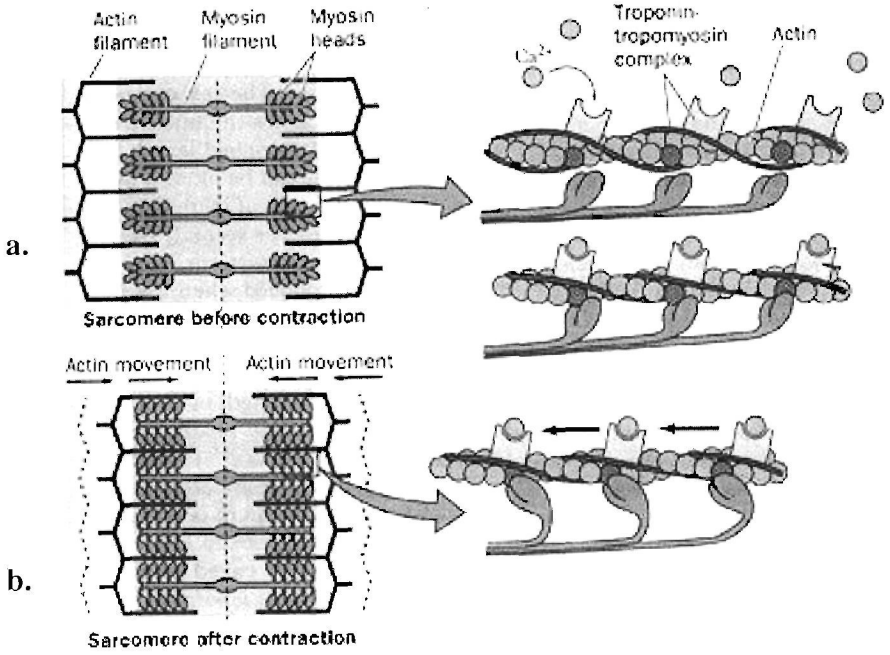
as obesity, diabetes, hypertension, smoking and poor dietary habits can accelerate the turn over and apoptotic death of endothelial cells to give abnormal endothelial function [Luscher & Vanhoutte, 1990]. In these cases, endothelial cells are not effective in producing bioavailable NO while the production of endothelium-derived vasoconstrictors is upregulated. These vasoconstrictors (usually eicosanoids), as well as oxidative lipoproteins and growth factors lead to the initiation of the atherosclerotic process and the characteristic morphological changes in the structure of the vessels.

1.3.1. Vasoconstriction and Vasodilation Pathways

Vascular smooth muscle contraction is described by the sliding-filament model that applies in all muscle contractions (smooth, skeletal and cardiac muscles). Within the sarcoplasm of the muscle cell there are subcellular elements and long prominent myofibrils, composed of bundles of contractile proteins. Each myofibril has many short structural units, known as sarcomeres, which are composed mostly of actin thin filaments and myosin thick filaments. Sarcomeres represent the minimal contractile unit of a muscle. Tropomyosin, a contractile accessory protein, covers the myosin binding sites on actin, inhibiting interaction of actin with myosin. The appearance of Ca^{+2} in the sarcoplasm leads to Ca^{+2} binding on troponin-C, a second contractile accessory protein, which moves the attached tropomyosin molecule deep into actin, uncovering the myosin binding sites on actin subunits. The exposed sites are then available to interact with myosin headpieces. When Ca^{+2} is removed from sarcoplasm the conformational states of troponin and tropomyosin prevent the actin-myosin interaction and lead to the relaxed

state. Actin and myosin filaments as well as the accessory proteins compose the contractile machinery of the vascular smooth muscle (Figure 1.2).

Figure 1.2. Relaxed (a) and Contracted (b) Contractile machinery (adopted by www.physioweb.org).



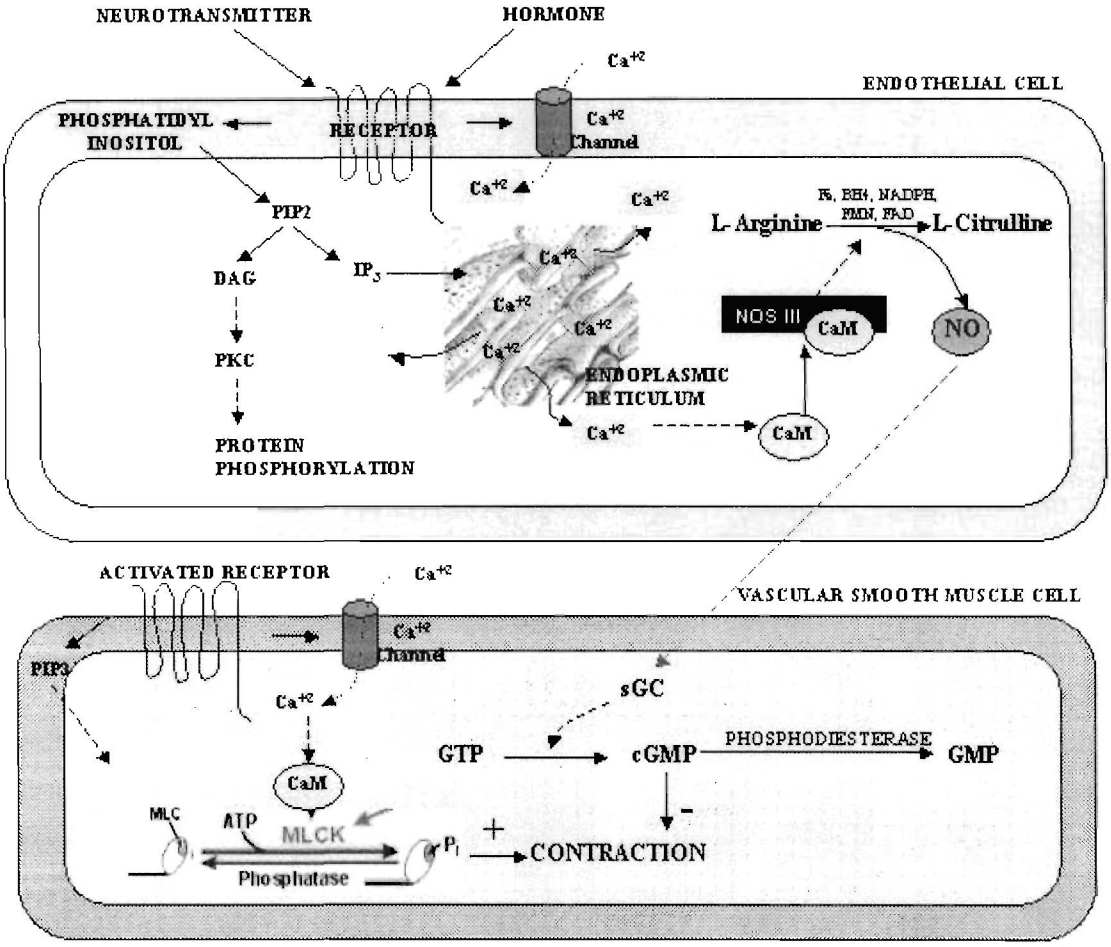
Vascular constriction and dilation are the result of vascular smooth muscle response to mediators that are circulating in the blood or are produced by the endothelial cell layer on vascular innervations. Endothelium-dependent vasodilation has been demonstrated in various vascular preparations in response to stimuli such as acetylcholine, bradykinin, histamine, adenine nucleotides, thrombin, substance P, Ca^{+2} ionophores and increased blood flow [Moncada et al., 1991]. The major EDRF, which induces vasodilation is NO [Furchgott & Zawadzki, 1980], this highly reactive and diffusible free radical which produces vasodilation via the activation of soluble guanylate cyclase (sGC) and elevation of cGMP [Lüscher & Vanhoutte, 1990]. Cyclic GMP

induces vascular relaxation by increasing Ca^{+2} efflux, decreasing the amount of Ca^{+2} released from intracellular stores, activating a protein kinase which stimulates cAMP-dependent Ca^{+2} extrusion across the vascular smooth muscle sarcolemma, and by inhibiting the breakdown of phosphatidyl-inositol in vascular smooth muscle [Lüscher & Vanhoutte, 1990]. Nitric oxide diffuses in the smooth muscle but also into the lumen of blood vessels to affect the permeability of the endothelial cells to macromolecules. Following the identification of NO as EDRF, scientific research has focused on the role of NO for the regulation of vasomotor tone, platelet aggregation, platelet and leukocyte adhesion, and vascular smooth muscle cell proliferation [Venema et al., 1999].

Binding of a ligand to its receptor in the endothelial cell activates the receptor and initiates a signaling cascade of events, which usually involves the interaction of several different enzyme systems. This activation induces an influx of Ca^{+2} into the cytoplasm of the endothelial cell which binds to calmodulin to activate cyclooxygenase and nitric oxide synthase, and lead to the release of EDHF (Figure 1.3.) [Vanhoutte, 2003]. Nitric oxide diffuses to the smooth muscle to increase the formation of cGMP from GTP.

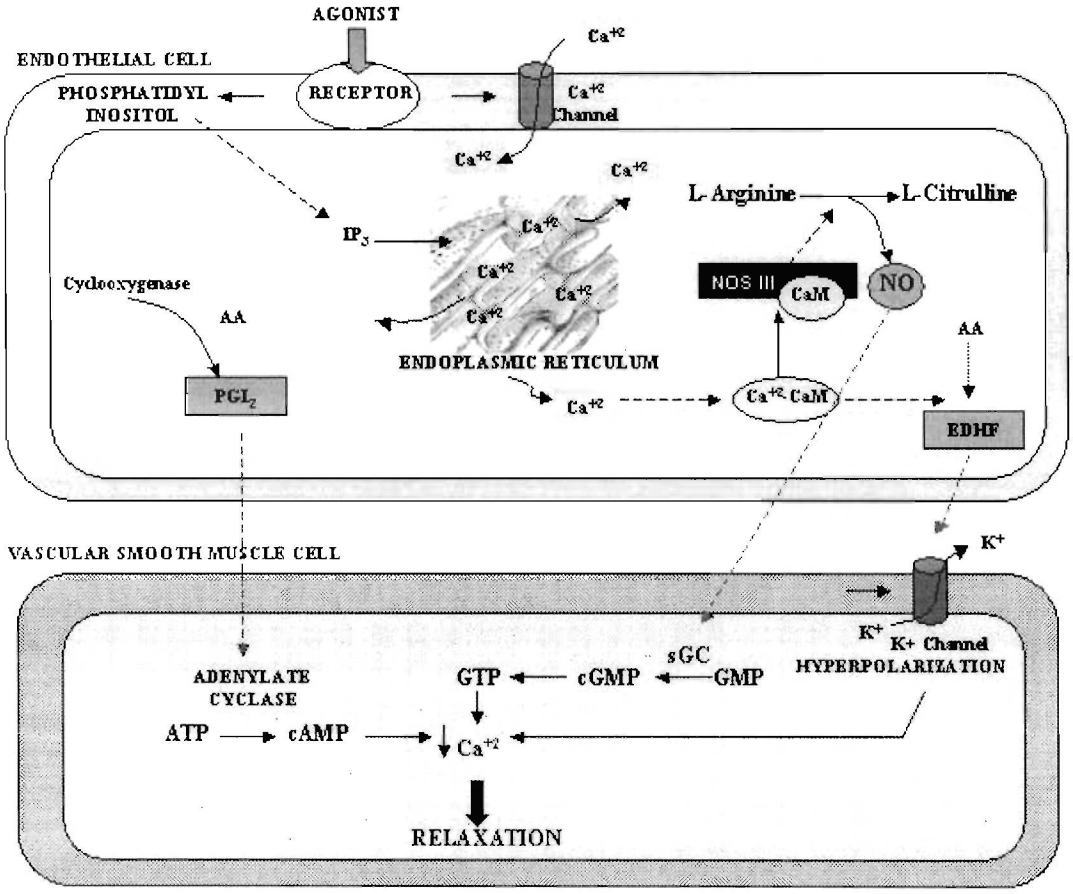
Endothelium-derived hyperpolarizing factor (EDHF) causes hyperpolarization by opening K^+ channels, and prostacyclin (PGI_2) activates adenylate cyclase (AC), which leads to the formation of cAMP. All of the above functions lead to vasodilation [Lüscher & Vanhoutte, 1990]. The contribution of hyperpolarization to endothelium-dependent vasodilation varies depending on the type of the vessel. Even though in large arteries, NO predominates under normal conditions, EDHF can mediate near normal levels of vasodilation when the endothelium is inhibited or is dysfunctional [Vanhoutte, 2003].

Figure 1.3. Vasoconstriction and vasodilation in endothelial and smooth muscle cell.



Adrenergic receptors are a heterogenous group of seven transmembrane domains/G-protein coupled receptors that mediate the actions of the natural adrenergic amines epinephrine and norepinephrine [García-Sáinz, 2000]. The α_1 adrenoreceptors stimulate phospholipase C activity, which catalyses the hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP_2) to diacylglycerol (DAG) and inositol 1,4,5,-triphosphate (IP_3). Diacylglycerol mediates the activation of protein kinase C and IP_3 acts a second messenger to mediate the release of intracellular calcium (Figure 1.4.) [Krauss, 2001]. The function of the receptor might be affected by cellular processes that alter its internalization, phosphorylation, degradation, regulation of expression or its binding to the ligands, such as the adrenergic agonist. Activation of the receptor leads to mobilization of intracellular calcium and takes place via two different mechanisms: Ca^{+2} influx through Ca^{+2} permeable channels and Ca^{+2} release from intracellular Ca^{+2} stores. Nitric oxide diffuses to the smooth muscle and acts through a cGMP-dependent protein kinase to control the phosphorylation and dephosphorylation of the myosin light chains (Figure 1.4.) [Lüscher & Vanhoutte, 1990].

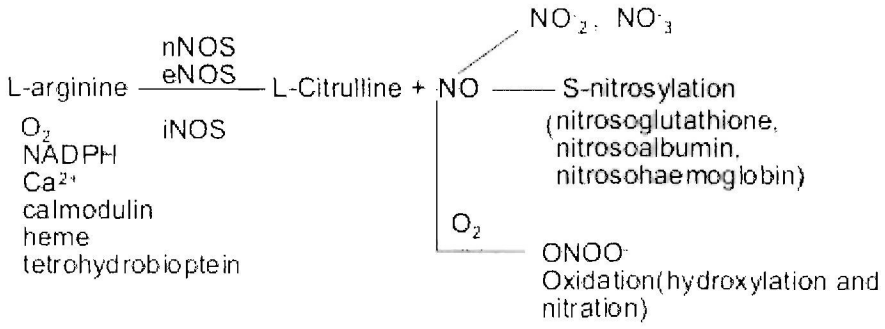
Figure 1.4. Endothelium-dependent vasodilation pathways.



1.3.2. Nitric Oxide

Mammalian tissues and various cell types, including the vascular cells, synthesize NO from L-arginine through a five-electron oxidation step (Figure 1.5). The substrates of NOS-mediated NO production are the amino acid L-arginine, molecular oxygen and NADPH [Michel et al., 1997]. The oxidation of L-arginine is catalyzed by a cytochrome P_{450}-type heme iron in the oxygenase domain of NOS. The electrons required for reduction of O_2 are shuttled from the cofactor NADPH to the heme, via a flavin-containing cytochrome P_{450} reductase that forms the C-terminal half of the NOS protein. Thus, cofactors that are required for NO generation are tetrahydrobiopterin (BH_4), flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). This electron transport chain only operates when Ca^{+2}/calmodulin is bound to the enzyme, which provides the Ca^{+2} regulation of endothelial NO synthesis [Mayer et al, 1998]. At low concentrations of L-arginine or in its absence, the enzymatic reduction of O_2 uncouples from substrate oxidation and results in the generation of superoxide anions and H_2O_2.

Figure 1.5. Nitric oxide generation from L-Arginine.



There are three isoforms of NOS which catalyze the formation of L-citrulline from L-arginine with the formation of NO: neuronal NOS (nNOS or NOS I) and endothelial NOS (eNOS or NOS III) which are constitutively expressed at low levels in a variety of cell types, and inducible NOS (iNOS or NOS II) which normally is not expressed in most cell types but can be induced for example by bacterial endotoxin and inflammatory cytokines [Wu & Morris, 1998]. NOS I and III are dynamically regulated by Ca^{+2}/calmodulin, while arginine serves as a substrate and acts structurally to facilitate the dimerization of NOS. Endothelial NOS is associated with caveolae at localized regions of the plasma membrane, and contains binding sites for heme and calmodulin, both being essential for enzyme activity. After Ca-loaded calmodulin is bound to NOS III between the C-terminal reductase and N-terminal oxygenase domain of NOS III, electrons are shuttled through the calmodulin-binding domain toward the heme-containing NOS III oxygenase domain, which results in the formation of citrulline and NO [Govers, 2001]. Unlike many other signaling molecules, ▪NO is freely diffusible, reacts with a diverse array of intracellular targets, and manifests biological effects that are dependent on the local chemical environment. Nitric oxide is a small lipophilic molecule with a biological half-life of several seconds; Thus, it can easily diffuse into and across plasma membranes and lipoproteins.

As a free radical, ▪NO readily reacts with other radical species and the metal centers of metalloproteins [Bloodsworth, 2000]. The metal-binding action of ▪NO to the heme iron of soluble guanylate cyclace (sGC) in the vascular smooth muscle cells stimulates the production of cGMP, which activates cGMP-dependent kinases and membrane ion channels to decrease intracellular calcium levels and allow smooth muscle

cells to relax. Thus, nitric oxide has profound cardiovascular effects, including the regulation of blood pressure. Compounds such as nitroglycerine and sodium nitroprusside imitate an endogenous nitrovasodilatory system by being converted to nitric oxide [Moncada et al, 1993]. The biologic effects of NO include its role as a potent vasodilator, neurotransmitter and cell-signaling effector molecule, but also include effects to increase vascular permeability, mediate immune responses and inhibit platelet aggregation.

1.4. Manganese and Regulation of Vasomotor Tone

It has been reported that Mn deficiency causes ultrastructural changes in the arterial wall, such as alterations in integrity of cell membranes, swollen and irregular endoplasmic reticulum, elongated mitochondria with crystal structures, and enlarged and abnormal Golgi apparatus [Freeland-Graves, 1994]. Previous studies from our laboratory have reported that Mn interferes in the metabolism of glycosaminoglycans, proteoglycan molecules that participate in the structure of the cell membrane [Yang et al, 1998a][Yang et al, 1998b][Ekanayake et al, 1995][Kalea et al, 2004]. Manganese is essential for preventing surface damage of the aorta and maintaining the density of the extracellular matrix around smooth muscle cells, especially in the medial layer [Yang et al, 1998]. Transmission electron microscopy of the arterial wall shows less dense extracellular matrix around smooth muscle cells, especially in the medial layers of Mn-deficient rats to suggest the possibility of changes in endothelial and/or vascular smooth muscle cells, which could affect the contractile properties of arteries [Ekanayake et al, 1995]. Manganese is involved in arterial glycosaminoglycan (GAG) metabolism by affecting the total proteoglycan content

of the aorta, and by altering the molecular weight and sulfation pattern of chondroitin sulfate in the same tissue [Yang et al, 1998a][Yang et al, 1998b]. In addition, Mn concentration in the diet affects the heparan sulfate sulfation pattern and disaccharide composition in Sprague-Dawley rats to suggest the possibility of Mn-mediated alterations in cellular signal transduction pathways [Kalea et al, 2004]. Changes in vascular GAG molecules might predispose vessels to lipid deposition, lipoprotein oxidation and cardiovascular disease.

Animals deficient in Mn have decreased activity of MnSOD in the heart, liver and kidney [Ensunsa et al, 2004][Malecki et al, 1994][Malecki et al, 1996]. However, there are no studies to our knowledge on the MnSOD activity in vascular tissue. If MnSOD activity is reduced in vascular tissue, which is possible in severe Mn deficiency, we would expect a compromised antioxidant defense system in the vascular cell with possible increase in oxidative stress. An increase in the production of superoxide anions would reduce the bioavailability of NO and increase the production of its by-products (peroxynitrite ONOO-, nitrite NO_2 and nitrate and NO_3), which could affect cell-signaling pathways for vasodilation [Gao et al, 1994][Harris et al, 1995][Williams et al, 1998][Matsuoka et al, 2001].

There have been several studies on the *in vitro* effect of different acutely-applied concentrations of Mn on contraction of different vascular beds [Deth et al, 1981][Kuribayashi, 1969][Sullivan et al, 1968][Yan et al, 1998][Hoffman et al, 1981][Collins et al, 1972][Weiss et al, 1985], but the effect of dietary Mn on the functional properties of the arteries as described by vascular contraction and relaxation has not previously been studied. The *in vitro* effect of Mn on vascular contraction is related to the role of Mn on calcium transport mechanisms. The divalent structure of Mn enables it to compete with Ca^{+2} for binding anionic sites and to inhibit Ca transport [Deth

et al, 1981][Kuribayashi, 1969][Sullivan et al, 1968][Yan et al, 1998][Hoffman et al, 1981][Collins et al, 1972][Weiss et al, 1985]. Kasten et al. [Kasten et al, 1994] have reported that the addition of Mn *in vitro* potentiates NO-induced vasodilation, while a recent study [Ensunsa et al, 2004] has reported that the elimination of Mn from the diet enhances endothelium-mediated vasodilation. It has been suggested that severe Mn deficiency leads to lower activity of arginase and thus reduced competition with the endothelial NOS for L-arginine, the substrate that forms NO [Ensunsa et al, 2004]. Overall, the effect of different dietary Mn levels on vasodilator and vasoconstriction mechanisms are unclear.

In the normal state, the endothelium plays a major role in vascular homeostasis by regulating smooth muscle tone via potent vasoactive agents (NO, PGI_2 , EDHF, epinephrine, endothelin and thromboxane). We do know that chronic exposures to several modulatory influences can upregulate the release of endothelium derived relaxing factors to affect biomechanical properties of the arteries. Such influences include exercise, estrogens, increases in blood flow and dietary factors such as consumption of unsaturated fatty acids, red wine polyphenols and other antioxidants [Vanhoutte 2003][Mombouli et al., 1996]. Dietary factors such as Mn intake could also affect vascular function. Taking into consideration the multifunctional role of Mn as an essential cofactor in many enzyme systems as well as its effect on vascular structure, we hypothesize that Mn intake might affect the biomechanical properties of the arteries *ex vivo* as defined by the constriction and dilation of the vascular smooth muscle to various agonists.

1.5. Objectives and Significance of the Study

The goal of the present study was to explore the effect of dietary Mn on the biomechanical properties of the rat aorta through diets containing different Mn concentrations.

The objectives of this study were to determine the effect of various dietary concentrations of Mn on:

1. Endothelium-dependent and endothelium-independent vascular contraction as described by maximum force of contraction and vessel sensitivity in response to the α1 adrenergic agonist L-Phenylephrine in intact and endothelium disrupted aortic rings.

2. Endothelium-dependent and endothelium-independent vascular dilation as described by maximum relaxation and vessel sensitivity in response to the endothelium-dependent muscarinic agonist Acetylcholine and to the endothelium-independent nitric oxide donor Sodium Nitroprusside in intact aortic rings.

3. Endothelium-dependent vasodilation by inhibiting nitric oxide (NO) production (L-NMMA), by stimulating NO production through increased L-arginine substrate and by inhibiting the cyclooxygenase pathway (Mefenamic acid).

4. The expression of the two NOS isoforms (NOS II and III).

There have been no *ex vivo* studies to date on the effect of dietary Mn on both the vasoconstrictor and vasodilation pathways as related to regulation of vasomotor tone, vascular function, blood pressure and cardiovascular disease. Present knowledge comes

from studies on the structural effect of Mn on glycosaminoglycan structure of the rat aorta, which participate in several cell-signaling pathways by affecting the binding of ligands to receptors. There have been only a few recently published studies on the *in vitro* effect of Mn on constriction of vascular tissue but these have only limited information on the effect of Mn on NO-mediated vasodilation in rat aorta. In the present study we documented the effect of dietary Mn on several key effectors that participate in the cell signaling pathways for vasoconstriction and vasodilation.

Vascular ring studies are considered to be a broadly accepted way to study the role of the endothelium in vasomotor control and the effect of endothelial dysfunction on the biomechanical properties of the arteries, since in most cases the results match the observations in isolated human blood vessels [Vanhoutte, 1999]. Chapter 2 of this dissertation addresses the first objective and discusses how Mn affects adrenergic receptor mediated vasoconstriction. Chapter 3 addresses the second objective regarding the role of Mn on vasodilation pathways, and chapter 4 addresses the third and fourth objectives i.e. the effect of Mn on several pathways that are involved in vasodilation. Chapter 5 gives a summary of the results, the overall conclusions of the study and recommendations for future work.

Chapter 2

The Effect of Dietary Manganese on Endothelium-Dependent and Endothelium-Independent Receptor Mediated Vasoconstriction

In the present study we examined the effect of dietary manganese (Mn) on the vascular contractile machinery of rat thoracic aortas. Weanling male Sprague-Dawley rats were fed either a manganese deficient (MnD), adequate (MnA/ Control group) or supplemented (MnS) diet (<1, 10-15 and 45-50 ppm Mn respectively). After 15 weeks on the diet the rats were sacrificed and four aortic rings of 3 mm length were prepared. In two of the four aortic ring preparations the endothelial layer was mechanically disrupted while in the other two rings the endothelial layer was left intact. All aortic preparations were contracted by six cumulative doses of the α_1 adrenergic receptor agonist L-Phenylephrine (L-Phe, 10^{-8} to 3 x 10^{-6} M) under 1.5gm preload and were relaxed with one dose of Acetylcholine (Ach, 3 x 10^{-6} M) to confirm functional endothelium. The maximal force (Fmax) of contraction (vessel reactivity), as well as the vessel sensitivity (pD_2) to the adrenergic agonist were determined. Manganese deficiency, assessed by hepatic Mn content, significantly lowered the rate of animal growth.

In endothelium-disrupted rings, the Fmax of MnD animals was significantly lower than the Fmax of the MnA animals, while the Fmax of the MnS animals was significantly higher than the Fmax of MnA animals. In contrast, there were no statistically significant differences in vessel sensitivity (pD_2) of endothelium-disrupted rings among diet groups. These data indicate that Mn has relatively little effect on

membrane-related receptor-mediated mechanisms (no pD_2 effect) but does act to enhance intracellular vascular smooth muscle mechanisms at the level of the contractile machinery (Fmax effect).

In the endothelium-intact rings, MnS animals developed lower Fmax when contracted with L-Phe compared to the MnA animals ($p \leq 0.001$); however the vessel sensitivity (pD_2) of the MnS animals to the adrenergic receptor was not significantly different from the MnA animals. This suggests that Mn acts to increase an endothelium-derived relaxing factor on the contractile machinery, in order to blunt the Phe-induced contraction of vascular smooth muscle cells in MnS groups. Thus, dietary Mn at levels 45-50 ppm affects the contractile machinery by reducing maximal vessel contraction to an α_1 adrenergic agonist but appears to have little effect on membrane-related signal-transduction mechanisms. The Fmax in the MnD animals was not significantly different from MnA animals; however there was a significantly greater vessel sensitivity (pD_2) of the MnD group aortic rings to L-Phe when compared to the MnA/ Control group ($p \leq 0.001$). This suggests that there is smaller release or smaller effect of an endothelium-derived relaxing factor to give less (greater Fmax) blunting of the Phe-induced contraction of vascular smooth muscle cells in the MnD group. These data also suggest that there is a release of an endothelial factor to increase the sensitivity (pD_2) of membrane-related responses to the α_1-adrenergic receptor activation in the MnD group.

Our results demonstrate for the first time that dietary Mn influences the receptor signaling pathways and contractile machinery of vascular smooth muscle cells in response to an α_1 adrenergic receptor agonist.

2.1 Introduction

The crucial role of nutrition on the genesis and progress of cardiovascular disease (CVD) has been studied extensively, since cardiovascular disease still remains the primary cause of death in the U.S. population [American Heart Association report, 2003]. Trace minerals and among them Mn, may be potential risk factors for cardiovascular events, that cause and/or promote its development since it seems to participate in cell signal transduction pathways which in turn seem to affect the biomechanical properties of the vessels. The vascular endothelium mediates the relationship between cardiovascular risk factors and development of cardiovascular disease [Vogel, 1997][Vanhoutte, 2003]. Endothelial dysfunction is described as a condition where the endothelial release of relaxing factors is reduced and the secretion of contracting mediators is enhanced [Vanhoutte, 2002]. The role of endothelial dysfunction in hypertension, arteriosclerosis, and vascular diseases depends on a balance between the above factors [Russo et al, 2002][Cai et al, 2000][Shimokawa, 1999][John et al, 2000]. These vasoactive mediators are implicated in early inflammation and are known to promote or inhibit vasodilation, vasoconstriction, blood coagulation, thrombogenesis, thrombolysis, as well as endothelial cell growth and remodeling [Gao et al, 1994][Nilius et al, 2001][Boulanger et al, 1999][Mombouli et al, 1999]. Thus, an alteration in the biomechanical properties of the vascular system might affect not only blood flow, but also platelet aggregation and vessel permeability, processes that participate in the early stages of atherosclerosis and have been associated with hypertension, atherosclerosis and several cardiovascular disorders [Van Poppele et al, 2001][Drexler et al, 1999][Harris et al, 1995][Abeywardena et al, 2002][Chamiot-Clerc et al, 1999].

Several trace elements such as zinc, copper, selenium, magnesium, manganese (Mn), nickel, molybdenum and calcium are reported to affect the process of cardiovascular disease [Liu et al, 2002][Yan et al, 1998][Yan et al, 2001] by interacting with ion channels, neurotransmitters, receptors and/or ionic channel-receptor complexes [Nilius et al, 2001]. Previous studies have shown that Mn may affect blood pressure by decreasing the tension of isolated vascular tissue preparations *in vitro* [Kasten et al, 1994]. However, there have been few animal studies to associate the dietary deprivation of Mn with cardiovascular activity *in vivo* or *ex vivo* [Ensunsa et al, 2005].

Our laboratory was the first to report that Mn plays an important role in maintaining the integrity of blood vessels [Taylor et al, 1997][Klimis-Tavantzis et al, 1993][Yang et al, 1998][Yang et al, 1998b][Ekanayake et al, 1995]. Manganese is involved in arterial glycosaminoglycan metabolism and affects the total proteoglycan content of the aorta, alters the molecular weight and sulfation pattern of chondroitin and heparan sulfate in the same tissue, thus predisposes the vessel to lipid deposition, lipoprotein oxidation and cardiovascular disease [Kalea et al, 2005][Yang et al, 1998][Yang et al, 1998b]. Manganese is essential for preventing surface damage of the aorta and maintaining the density of the extracellular matrix around smooth muscle cells, especially in the medial layer [Ekanayake et al, 1995]. Transmission electron microscopy of the arterial wall revealed less dense extracellular matrix around smooth muscle cells, especially in the medial layers of the MnD rats, suggesting possible changes in the endothelial and/or vascular smooth muscle cells, which affect the contractile properties of the arteries [Yang et al, 1998][Yang et al, 1998b].

Vascular ring studies in animals are considered to be a broadly accepted way to experiment on the role of the endothelium in the vasomotor control and on the effect of

endothelial dysfunction on the biomechanical properties of the arteries, since in most cases the results match the observations in isolated human blood vessels [Vanhoutte, 1999]. Our knowledge on the role of Mn on arterial wall structure and the contractile properties of the arteries is limited and remains to be established. There have been no studies in the past on the effect of different concentrations of dietary Mn on the vascular contractile properties. In our study we examined the role of dietary Mn on arterial functional properties and on receptor sensitivity of Sprague-Dawley rat aortic rings, in the presence and in the absence of the endothelial layer, as assessed by aortic smooth muscle contraction in response to Phe, an α_1 adrenergic agonist.

2. 2. Materials and Methods

2.2.1. Animal Model

Thirty weanling male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were randomly assigned to three groups of ten rats, each on a Mn deficient (MnD), Mn adequate (MnA) and Mn supplemented (MnS) diet with <1ppm, 10-15ppm and 45-50 ppm of Mn respectively. The above Mn levels were chosen because studies indicate that a Mn level of less than 1.0 ppm results in Mn deficiency in about thirteen to fifteen weeks [Taylor et al, 1997][Klimis-Tavantzis et al, 1993][Yang et al, 1998][Yang et al, 1998b]. Ten ppm dietary Mn levels have been reported by the American Institute of Nutrition as an adequate level to prevent deficiency in the rat [Reeves et al, 1993], thus MnA diet group was used as a Control group. The animals were individually

housed in metal mesh-bottomed cages in an environmentally controlled room maintained at 22°C with a 12:12 hrs light:dark cycle. All animals were weighed weekly. The Animal Care and Use Committee of the University of Maine approved all animal care and experimental procedures.

2.2.2. Diets

Diets were mixed in our laboratory from purified ingredients (Table 2.1), as described before [Yang et al, 1998][Yang et al, 1998b]. Vitamin (A.O.A.C. Special Vitamin Mixture, Harlan Tekland) and mineral mixes (ICN Biochemicals, Cleveland, OH) were commercially prepared. Manganese content of the diets and tap water were determined 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 ppm). Diet content of Mn was tested every time following diet preparation. Food consumption was measured daily in all animal groups. Tap water (below the detection limit for Mn) and food was provided *ad libitum*.

2.2.3. Tissue sampling

At the end of the feeding period, rat food was withheld for 12-14 hrs. Animals were anesthetized in a chamber with 95% CO_2 / 5% O_2, for approximately 2 min. Thoracic aortas and livers were removed carefully and washed with physiologic salt solution (PSS, with composition in mmol/L: NaCl, 118, KCl, 4.7, $NaHCO_3$, 25, KH_2PO_4, 1.18, $MgSO_4$, 1.17, Dextrose, 11, $CaCl_2$, 1.25). Liver and kidney tissues were weighted,

lyophilized, pulverized and analyzed for Mn content 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.

2.2.4. Vascular Ring Studies

2.2.4.1. Aortic Preparations

The thoracic aorta was removed and submerged in a petri dish filled with physiologic salt solution (PSS) at room temperature. The aortic segment was cleaned of adherent fat and of connective tissues and four rings of 3 mm length were prepared from the middle part of the vessel. Two of the four rings were left intact and the other two were disrupted from the endothelial cell layer as described below (2.2.4.2). Two stainless steel wire triangles (0.012in diameter) were passed carefully through each ring. Each aortic ring was mounted in a 20ml Radnotti tissue bath, which contained PSS maintained at 37°C by a thermoregulated water circuit and was continuously bubbled with a 95% O_2 and 5% CO_2 mixture in order to keep the pH at 7.4. The aortic ring was attached through the one triangle to a fixed glass hook in the tissue bath and through the other triangle to a force transducer, which was connected to a tissue force analyzer (Model 410, MicroMed, Louisville, KY) for the measurement of isometric force. Changes in force were continuously displayed and recorded on a personal computer through the use of a system integrator software program (DMSI-210 Version 1.01, MicroMed).

2.2.4.2. Mechanical Disruption of the Endothelial Cell Layer

Endothelial disruption was obtained in two of the four aortic rings from each animal by gentle rubbing of the aortic intima as described previously by Vanhoutte [Luscher & Vanhoutte, 1990]. The aortic ring was rolled smoothly with the curved tips of a stainless steel forceps on a PSS saturated paper towel as shown in Figure 2.1.

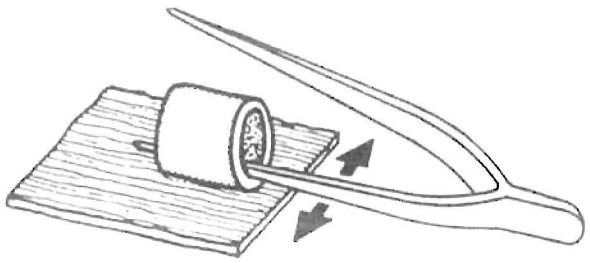


Figure 2.1. Mechanical disruption of the endothelium.

2.2.4.3. Experimental Protocol

A total of 120 intact rings (60 intact and 60 endothelium disrupted aortic rings from each diet group, ten animals in each diet group) were used to study the receptor-mediated vasoconstriction. Resting tension (preload) was adjusted to 1.5g, since this preload provides the optimum concentration-response relationships to a variety of agonists, including the α_1 adrenergic receptor selective agonist L-Phe [Angus et al, 2000][Asbun-Bojalil et al, 2002][Adeagbo et al, 1993][Beach et al, 2001][Kawabe et al, 2003]. Tissues were allowed to equilibrate for 60min. During this time, all rings were washed three times with aerated PSS (37°C, pH 7.4) and were precontracted for 10min with one dose of L-Phe (10^{-8} M) and one dose of Ach (10^{-8} M), in order to eliminate non-

specific tissue binding for the agonists. At the completion of this preconditioning dose the rings were washed four times for 20min with aerated PSS and the tension (preload) was corrected to the original baseline levels and remained unchanged throughout the experiment.

Each ring was contracted with cumulative applications of six concentrations of L-Phe (in 3 X steps) over the range (10^{-8} to 3 x 10^{-6} M) as it has been described before [Kalea et al, 2005]. A drug-tissue contact time of six minutes was allowed for each L-Phe concentration to achieve the maximum contraction. The presence of viable endothelium was assessed in all preparations by determining the ability of Ach (Ach, 3 x 10^{-6} M) to induce more than 70% of vasodilation in the presence of L-Phe. Rings were washed four times over 25 minutes with PSS (37°C, PH 7.4), after each agonist treatment, to bring aortic tension down to or slightly below the original preload level. A second L-Phe dose response curve was obtained, followed by the same dose of Ach, under the same passive tension (preload) in order to duplicate the contraction curve.

2.2.5. Drugs

Acetylcholine (Ach) chloride, L-Phenylephrine (L-Phe) and salts for the stock solutions of the physiologic salt solution (PSS: NaCl, KCl, $NaHCO_3$, KH_2PO_4, $MgSO_4$, Dextrose, $CaCl_2$) were purchased in pure forms from Sigma-Aldrich Chemical Co (St Louis, MO, U.S.A.) and were dissolved in distilled water.

2.2.6. Statistical Analysis

Animal body, liver and kidney weights as well as liver and kidney Mn concentration were compared using a one-way analysis of variance (ANOVA). The mean daily food intake was measured in a previous pilot experiment [Kalea AZ et al 2005] in which we observed no significant differences in food intake among animals from different diet groups. Thus, food intake was not measured in this experiment.

In intact rings, relaxation to the Ach dose lower than 70% was an indication of possible damage in the endothelium during the experimental procedure and was used as a qualitative exclusive criterion (intact rings with >70% of Ach relaxation were excluded). Similarly, in endothelium-disrupted rings relaxation to the Ach dose higher than 5% was used as an indicator of incomplete mechanical disruption of the endothelial cell layer thus, these rings were also excluded.

Contractile responses were expressed as the force derived (g) in response to the maximal dose of L-Phe (Fmax). Maximal contraction force (Fmax) was an indicator of changes in contractile machinery events such as the myosin light-chain kinase and actin-myosin cross bridge activity [Pratt & Taylor, 1990]. Comparisons of the maximal contraction force (Fmax) developed in all intact and disrupted rings revealed differences of the contractile machinery among animals with different dietary Mn intake. Half-maximal response (EC_{50}) values for L-Phe were extrapolated from the dose-response curves. Using the EC_{50} data, we estimated the vessel sensitivity to L-Phe as described by the pD_2 value (pD_2=-$\log_{10}$ EC_{50}). The pD_2 values for the intact and denuded rings of all animals also showed differences in the vessel sensitivity to the α_1 adrenergic receptor among diet groups.

All results were expressed as mean values ± standard error of the mean (SEM). The Fmax and pD_2 values to L-Phe were compared in two-way analysis of variance (ANOVA) tests in order to determine the effect of diets on the contractile machinery (Fmax) and on vessel sensitivity (pD_2). Student Newman-Keuls multiple comparison tests were used for statistical analysis and a p-value level of 0.05 or less was considered as statistically significant. The Sigmastat Statistical Program Package (SAS Institute, Cary, NC) was used to conduct the statistical analyses.

2.3. Results

2.3.1. Animal Growth

All animals fed a MnD, MnA or MnS diet gained weight. However, mean body weights were significantly different among all groups (Table 2.1). The rate of growth in Mn deficient (MnD) animals was slower compared to the Mn adequate (MnA) and Mn supplemented (MnS) rats (Table 2.1). The difference in growth rate was statistically significant starting the fourth week after the initiation of the diet as it has been reported in the past [Yang & Klimis, 1998a]. Kidney weights (Table 2.1) were not statistically significant among diet groups while liver weights of the MnD rats were reduced when compared to MnA. This reduction was proportionate to the total body weight reduction. The percentage of liver weight to body weight of the rats was not different among the diet groups (Table 2.1). Manganese deficiency was confirmed by hepatic and kidney Mn

concentration, which significantly decreased only in the MnD group (Figures 2.1 and 2.2).

Table 2.1. Effects of dietary Mn on body, liver and kidney weight.

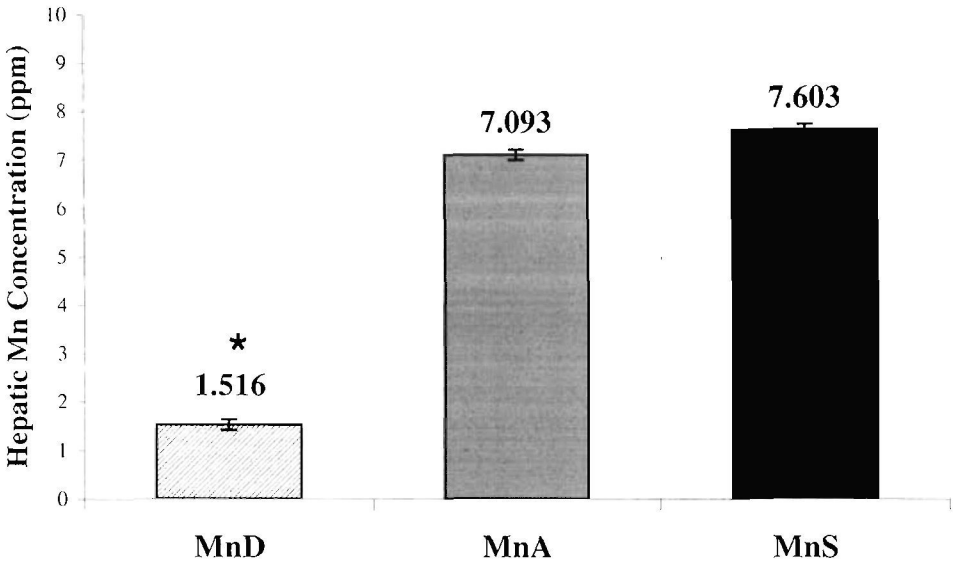
Diet Groups	Body weight (g)	Liver weight (g)	Liver weight as a % of body weight	Kidney weight (g)
MnD	385 ± 11*	11.46 ± 0.45*	3.0 ± 0.001	3.45 ± 0.10
MnA	468 ± 12	14.39 ± 0.60	3.1 ± 0.001	3.50 ± 0.10
MnS	474 ± 11*	14.00 ± 0.55*	3.0 ± 0.001	3.68 ± 0.08

(*) Statistically significant differences at $p \leq 0.05$ compared to MnA/ Control group.

2.3.2. Response to L-Phenylephrine-Induced Vasoconstriction

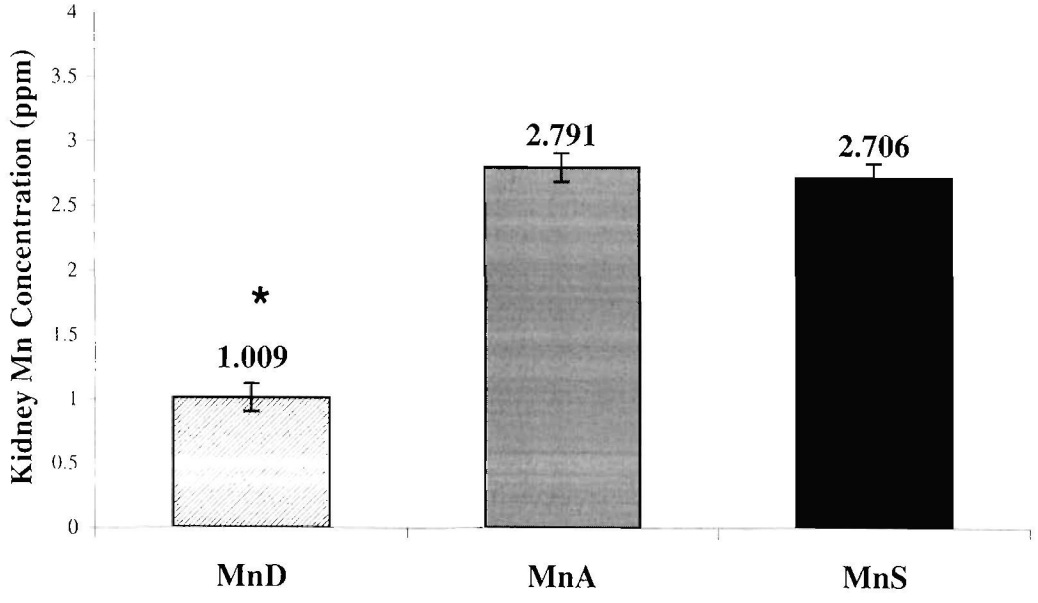
Six cumulative doses of L-Phe were used to give concentrations of 10^{-8}, 3 x 10^{-8}, 10^{-7}, 3 x 10^{-7}, 10^{-6} and 3 x 10^{-6} M in the tissue baths to produce graded increases in concentration force. The maximum contraction force developed in response to L-Phe was observed at drug doses higher than 10^{-6} M (5^{th} and 6^{th} L-Phe doses). Washout of agonist over 25 min reduced vasoconstriction at least to the preload value.

Figure 2.2. Hepatic Manganese Concentration among diet groups.



(*) Statistically significant differences at $p \leq 0.001$.

Figure 2.3. Kidney Manganese Concentration among diet groups.



(*) Statistically significant differences at $p \leq 0.001$.

The decrease in tension to the 3 x 10^{-6} M Ach dose was divided by the ring tension just prior to the Ach dose (point after the last point of the final L-Phe dose) and was used to calculate a percent relaxation for that ring when the drug was applied. Relaxations to the one dose of Ach were expressed as percentages of the level of final precontraction. Application of the Ach dose at the end of the L-Phe concentration response curve significantly relaxed the L-Phe contracted aortic rings of all groups (approx. >70%) and that indicated that the endothelium was not damaged during the tissue extraction process and the aortic ring preparation. All endothelium-disrupted rings failed to relax in response to Ach (<5%).

As expected the Fmax values in endothelium-disrupted rings was greater when compared to the intact Fmax values developed in each diet group (Figure 2.4). The influence of dietary Mn on the effect of endothelial cell to alter maximum response (Fmax, mean value ± SEM) to the adrenergic agonist (L-Phe) is presented in Figure 2.5. In endothelium-disrupted rings the maximum force of contraction (Fmax) was increased significantly following the increase in Mn concentration in the diet (Figure 2.4). The Phe-induced constriction in the vascular smooth muscle (VSM) as described by the Fmax of disrupted rings was reduced MnD-fed animals and enhanced in MnS-fed animals when compared to the MnA/ Control group.

In the presence of endothelium (endothelium-intact rings) the force developed in MnD-fed animals was not significantly different when compared to the Control group (Figure 2.4). However, aortic rings from animals supplemented with 45-50 ppm of Mn (MnS group) developed significantly lower Fmax when compared to both MnD and MnA animals (Figure 2.4). Supplementing the diet with 45-50 ppm of Mn did not change the

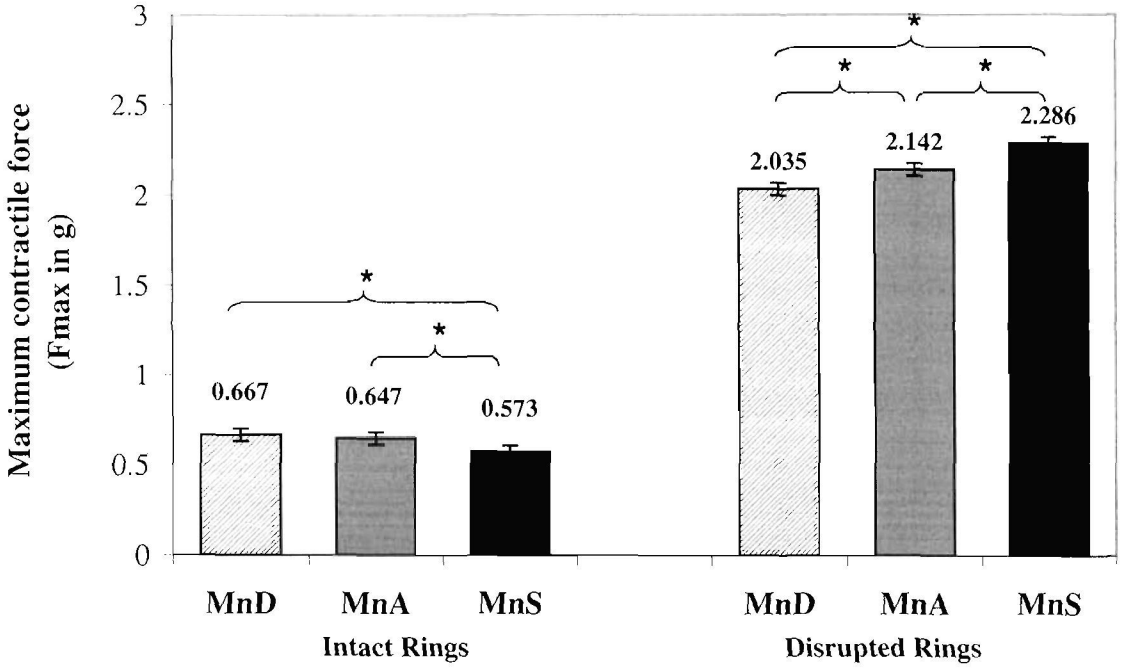
stores of Mn in the body as described by hepatic and kidney Mn concentration (Table 2.1) but it did reduce the contractile responses to the α_1 adrenergic agonist (Phe) in the rat aortic rings in comparison to the MnA (10-15 ppm).

The effect of the presence of the endothelial cell is represented as the difference in Fmax between intact and disrupted rings. In MnD animals we observed a decreased effect or diminished role of the endothelium in the Phe-induced contraction of the aorta, when compared to the Control group (MnA). In MnS animals the effect of the endothelium on vasoconstriction was significantly enhanced.

The mean maximum contraction force for each ring was used to calculate a percent increase of force in each dose, in order to determine the EC_{50} value (the effective concentration of L-Phe in which 50% of maximum contraction was obtained). The negative log (base 10) of the EC_{50} value was calculated to give us the pD_2 value for each ring, a measure of vessel sensitivity to the α_1 adrenergic receptor response.

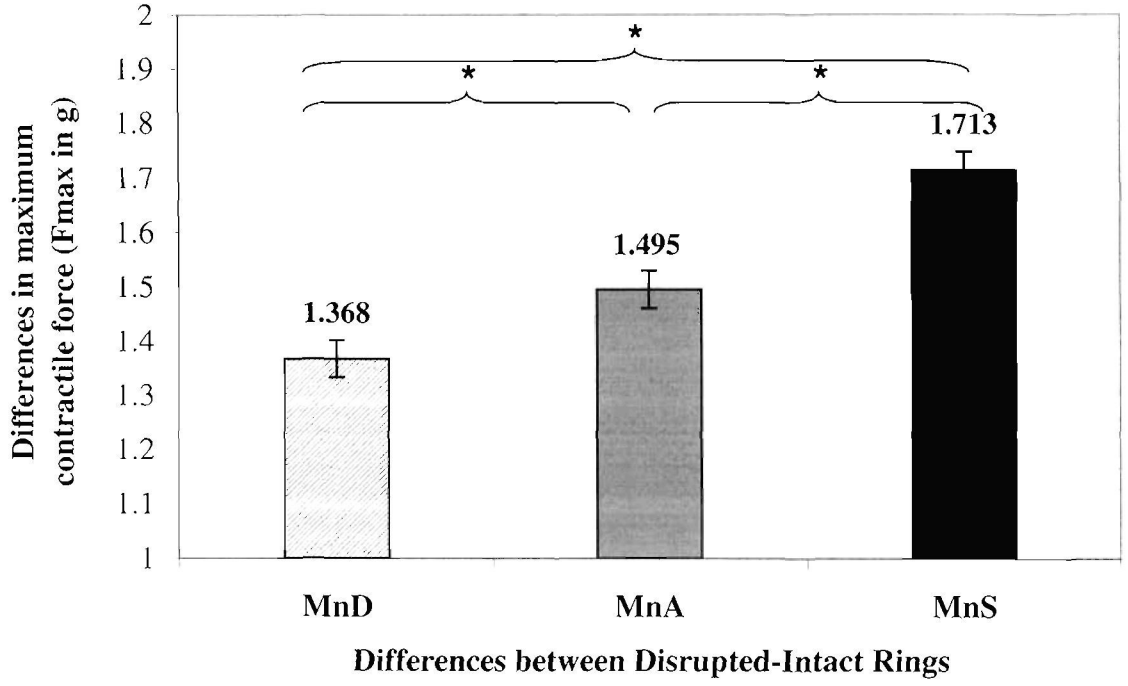
Vascular sensitivity (pD_2) values for intact aortic ring contractions to L-Phe were significantly greater in MnD animals when compared to Controls (Figure 2.6). The vascular sensitivity of MnA and MnS animals did not appear to be significantly different. In the absence of endothelium, the vascular sensitivity to L-Phe appeared to be similar among diet groups. Thus, restriction of dietary Mn below the adequate levels (10-15 ppm) appears to increase the vascular sensitivity to the α_1 adrenergic receptor only in the presence of endothelium.

Figure 2.4. The effect of dietary Mn on maximum response (Fmax, mean value ± SEM) to the α_1-adrenergic agonist (L-Phe) in endothelium-intact and endothelium-disrupted aortic rings in Sprague Dawley rat.



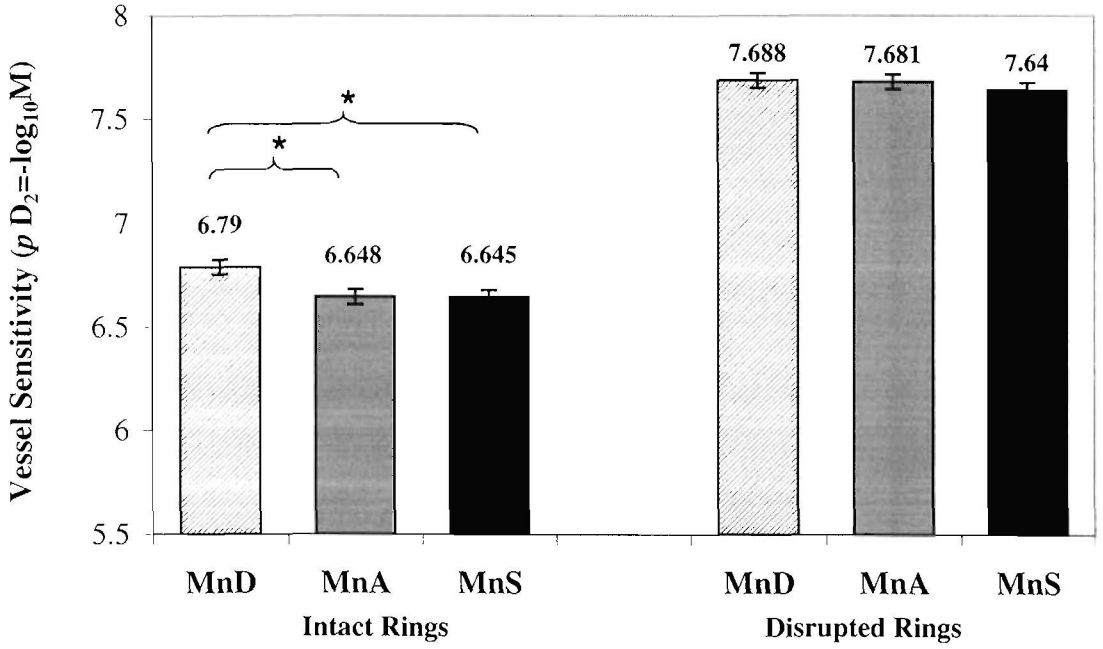
(*) Statistically significant differences at $p \leq 0.001$.

Figure 2.5. Influence of dietary Mn on the effect of endothelial cell to alter maximum response (Fmax, mean value ± SEM) to the adrenergic agonist (L-Phe) in Sprague Dawley rat.



(*) Statistically significant differences at $p \leq 0.001$.

Figure 2.6. The effect of dietary Mn on vascular sensitivity (pD_2, mean value ± SEM) to the α_1- adrenergic agonist (L-Phe) in endothelium-intact and endothelium-disrupted aortic rings in Sprague Dawley rat.



(*) Statistically significant differences at $p \leq 0.001$ compared to MnA/ Control group.

2.4. Discussion

Manganese deficiency was confirmed by the suppression of animal growth in the MnD diet group, as well as by the lower hepatic Mn content (Table 2.1). Retardation of rodent growth in Mn deficiency has also been reported by previous studies [Kalea et al, 2005][Taylor et al, 1997][Klimis-Tavantzis et al, 1993][Yang et al, 1998a][Yang et al, 1998b]Paynter, 1980][Fahim et al, 1990][Malecki et al, 1994] and it seems to be an effect of reduced efficiency of food conversion with the dietary Mn depletion [Yang et al, 1998a][Yang et al, 1998b]. The decrease in liver weight in MnD rats was proportional to the decrease in body weight in MnD animals as shown by the similar ratio of liver to body weight (Table 2.1). Kidney weight was similar among diet groups (Table 2.1). Manganese concentrations in liver and kidney were significantly reduced in the MnD diet group when compared to Control, while there were no differences among MnA and MnS animals (Table 2.1).

We document for the first time that the presence of Mn in the diet affects the Phe-induced contractions of the rat aortic vessel. The effect of the trace element included both the maximal contractile response to Phe and the membrane-related receptor sensitivity. Altering the Mn from control levels in the experimental diet, modified the above responses, which were affected by the presence of endothelium.

In endothelium-disrupted aortic rings, dietary Mn enhanced vasoconstriction on an α_1 adrenergic agonist (L-Phe) (maximum contraction: MnD < MnA/ Control < MnS). We observed an increased responsiveness of the vascular smooth muscle cells to the adrenergic agonist, which was independent from the receptor-agonist interaction (no differences in pD_2 values) when compared to the MnA/ Control group. Additionally,

when Mn was supplemented in 45-50 ppm the vessel sensitivity (pD_2) to the adrenergic agonist L-Phe was unaffected in both ring preparations (intact and disrupted); thus, supplementation of Mn in these levels does not affect the sensitivity of the adrenergic receptors in endothelial cell and vascular smooth muscle cell.

In the presence of endothelium, we observed an increase in adrenergic receptor sensitivity (higher pD_2) with Mn deficiency when compared to Control diet group. This increase was not accompanied by any significant difference in the responsiveness of the contractile machinery (Fmax). Intact aortic rings from MnS (45-50ppm) animals demonstrated a marked decrease in responsiveness of the contractile machinery to L-Phe as demonstrated by a lower Fmax, which cannot be attributed to a decrease in vascular sensitivity to the adrenergic agonist (no significant differences in the pD_2 values between MnA and MnS groups). When the endothelial cell was disrupted, the responsiveness of the MnS rings to vasoconstriction was higher (increased Fmax), when compared to MnA but also to MnD rings. That is an indication that the hypocontractility of the MnS aortic rings and is attributed to the increased vasodilator tone of the endothelial cell layer, which balances the increased constriction of the vascular smooth muscle (as observed in disrupted rings). We conclude that Mn affects endothelial cells blunting the enhancement of Phe-induced vasoconstriction caused by the presence of Mn in VSM. The enhanced effect or role of Mn on the contractile efficiency of the VSM is not on the receptor level but rather on the contractile machinery.

In rat aorta the contractile responses to selective α_1 adrenergic agonists are modulated by nitric oxide, which means that the absence of the endothelium will markedly potentiate the response to the agonist [Lüscher & Vanhoutte, 1990]. In the case

of L-Phe which does not evoke a rise in cGMP, the effects of the endothelium removal is due to basal rather than stimulated release of endothelium-derived relaxing factor(s).

In all our endothelium disrupted aortic rings we do observe a potentiation of the L-Phe-induced vasoconstriction in the presence of Mn. We know that the presence of a functional endothelium depresses the contractile actions of adrenoreceptor agonists [Allan et al, 1983][Carrier & White, 1985][Adeagbo & Triggle, 1993] an action attributed to the spontaneous release of endothelium-derived relaxing factor(s) and physiological antagonism of the contractile process. Endothelial disruption eliminates the role of endothelium in vascular contraction and thus augments vascular contractions to a variety of agonists [Martin et al, 1988]. Thus, in disrupted aortic rings we observe an enhanced response to vasoconstrictors, such as Phe, and a reduced relaxation to vasodilators such as Ach. These modified responses have been studied in different techniques of mechanical removal of the endothelial layer (Molina, 1992). The effect of the endothelium seems to be more pronounced in the MnS animals when compared to MnA and less pronounced in MnD animals when compared to the MnA/ Control animals (Figure 2.4).

In our study we examined vascular responsiveness to adrenergic stimulation of animals fed diets with different concentrations of Mn. We examined the α_1 adrenergic receptor mediated vasoconstriction using L-Phe, a synthetic catecholamine that causes VSM contraction. Phenylephrine binds to and activates the G-protein type α_1 adrenergic receptor, which leads to the hydrolysis of phosphoinositol biphosphate (PIP_2) to inositol triphosphate (IP_3) and diacylglycerol (DAG). Inositol triphosphate stimulates the release of calcium from intracellular stores, which acts as a second messenger and initiates a

cascade of events that lead to vascular smooth muscle contraction. Dietary Mn is a multifunctional trace element that may interfere in several different ways in that cascade of events and thus may be responsible for modulating vascular tone.

Studies *in vitro* have shown that when Mn is present in small concentrations (0.3-3μmol/L), it inhibits the vascular contractions induced by noradrenaline, 5-hydroxytryptamine and potassium in porcine coronary artery, goat and human cerebral arteries and in rat aorta [Yan et al, 1998][Yan et al, 2001]. In our study, we observed the effect of dietary intake of Mn on the Phe-induced contractions in the Sprague-Dawley rat aorta and confirmed the suppressor effect of Mn on α_1 adrenergic agonist induced-vasoconstriction of intact rings *ex vivo*. Our results showed that dietary Mn shifted the Phe dose-response curves to the right with a significant reduction in the maximum contractile response at Mn levels higher than 10-15 ppm. The exact mechanism involved in such inhibitory action of Mn is still unknown. Interestingly, this shift occurred when dietary Mn was supplemented (45-50 ppm) even though we observed no difference in the hepatic or kidney Mn concentration between the MnA and MnS groups (Table 2.1) suggesting that the contractile mechanism is more sensitive than the storage index for Mn.

Previous in vitro observations support several hypotheses that might explain the role of Mn in suppressing agonist-induced vasoconstriction [Liu et al, 2002][Yan et al, 1998][Yan et al, 2001][Salaices et al, 1983][Kuribayashi, 1969][Sullivan et al, 1968]. One of these hypotheses is 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} [Yan et al, 1998][Yan et al, 2001][Kuribayashi, 1969][Clarkson, 1993][Deth et al, 1981].

A complete depletion of the intracellular Ca^{+2} pool is known to activate Mn^{+2} non-permeable Ca^{+2} influx from the extracellular space in portal vein smooth muscle cells, in the presence of an adrenergic receptor agonist (in our case L-Phe) [Pacaud et al, 1993][Kwan et al, 2003]. This observation may explain the reduction in responsiveness of the contractile machinery of the rat aorta in the presence of dietary Mn >45 ppm as well as our finding that the hypocontractility of intact MnS rings is independent from the vascular sensitivity of the aorta to the adrenergic agonist. This hypothesis would not explain the enhanced vasoconstriction of VSM in the presence of Mn. Such an enhancement in maximum force developed indicates that the presence of Mn enhances the efficiency of the contractile machinery in the VSM and could imply an effect of the trace element on the cross bridge formation.

Manganese is known to affect the synthesis of metalloenzymes that protect the cell membrane structure from oxidative stress, such as Mn superoxide dismutase [Malecki et al, 1994][Zanetti et al, 2001][Wambi-Kiesse et al, 1999]. The presence of free radicals (O^{2-} generation) represents an important mechanism for endothelial dysfunction by reducing the bioavailability of the endothelium-derived relaxing factor nitric oxide (NO) [Gao et al, 1994][Williams et al, 1998][Matsuoka, 2001]. Ensunsa et al. [Ensunsa et al, 2002] have reported lower levels of liver endothelial nitric oxide synthase (NOS III) in Mn deficient animals and increased production of peroxinitrite products, which in turn may have an effect on the bioavailability of nitric oxide in the endothelial cell layer [Harris et al, 1995]. Decreased bioavailability of endothelial-released nitric oxide is associated with decreased vasorelaxation and may affect the biomechanical properties of the contractile machinery.

In our study we did not observe any differences in vasoconstriction of MnD rings when compared to Control rings, even though there was an increased vascular sensitivity to the adrenergic agonist (higher pD_2 of intact MnD rings but not of disrupted). However, we did observe a decreased constriction of the VSM in MnD rings only, when compared to the Control group, which was independent of the vascular smooth muscle cell sensitivity. This may suggest an impaired ability of the endothelial cell in MnD animals to synthesize vasodilators or protect their integrity in order to counterbalance vasoconstriction, which may be explained by the increased oxidative stress and decreased NO bioavailability in case of Mn deficiency.

In addition to a role for Mn in the contractile machinery of the vascular smooth muscle, our results show an increase in pD_2 values in the MnD group in intact rings only (Figure 2.5). These results occurred without a change in Fmax (Figure 2.5), we suggest that there is an increase in α_1 adrenergic receptor sensitivity of the endothelial layer during dietary Mn deficiency. Since Mn participates in the structure of the extracellular cell matrix (ECM) by affecting the synthesis and structure of certain glycosaminoglycan molecules (chondroitin sulfate and heparan sulfate) [Kalea et al, 2005][Yang et al, 1998][Yang et al, 1998b] and affects their sulfation pattern, it might also modulate agonist actions or interfere in the agonist-receptor binding or affect the receptor structure itself [Edwards et al, 1988][Strott, 2002][Lyon et al, 1998]. It has been reported that glycosaminoglycan molecules act as cellular co-receptors in order to facilitate the binding of receptors with ligands such as growth factors (VEGF, FGF etc) and lipoproteins [Kramer et al, 2003]. These cell surface receptor-ligand complexes are involved in the process of cellular signal transfer by affecting the responsiveness of the cellular

membrane to extracellular ligands, especially at low ligand concentrations [Risler et al. 2002]. Previous transmission microscopy studies conducted in our laboratory, reported a decreased density of the endothelium cell layer in Mn deficiency that may affect either the adrenergic receptor number or affinity [Ekanayake & Klimis, 1995]. However we did not measure directly the receptor number, affinity or occupancy.

Despite the expanding plethora of studies on the role of nutrients on the biomechanical properties of the vasculature, there are still many gaps on defining the exact signaling pathways and second messengers that are implicated to regulate contraction and relaxation within the cell. It has been reported that approximately only one third of all cardiovascular events can be extrapolated on the basis of "accepted" risk factors. Additional determinants may exist and trace minerals may be potential candidates.

Manganese is a multifunctional trace element participating in many fundamental processes in the cell [Korc, 1993]. Its effect on the vascular system, such as the control of vasomotor tone and blood pressure regulation still remains to be unraveled. Our results demonstrate, for the first time, that dietary Mn is implicated in the vascular contractile model, affecting the mechanical properties of blood vessels and regulating vascular tone. This effect depends on its concentration in the diet and on the presence of a viable endothelium.

Chapter 3

The Effect of Dietary Manganese on Endothelium-Dependent and Endothelium-Independent Vasodilation

In the present study we examined the effect of dietary manganese (Mn) on the vasodilation of rat aorta. Weanling male Sprague-Dawley rats were fed either a Mn deficient (MnD), adequate (MnA/ Control) or supplemented (MnS) diet (<1, 10-15 and 45-50 ppm Mn respectively). Following 14 weeks on the diet the rats were sacrificed and 3 mm aortic rings were prepared. After equilibration and preconditioning, aortic rings were precontracted with one dose of the α_1 adrenergic receptor agonist L-Phenylephrine (L-Phe, 10^{-6} M). Cumulative applications of seven concentrations of Acetylcholine (Ach, 3 x 10^{-9}, 10^{-8}, 3x 10^{-8}, 10^{-7}, 3x 10^{-7}, 10^{-6}, 3x 10^{-6}M) were applied to induce endothelium-dependent vasodilation. A second concentration response curve was applied after the same precontraction, using eight doses of sodium nitroprusside (SNP, 3x 10^{-9}, 10^{-8}, 3x 10^{-8}, 10^{-7}, 3x 10^{-7}, 10^{-6}, 3x 10^{-6}, 10^{-5}M) to assess endothelium-independent vasodilation. The percentage of relaxation to the initial precontraction was used to determine the vessel sensitivity (pD_2) to Ach and SNP.

Manganese deficiency, assessed by hepatic Mn content, significantly lowered the rate of animal growth. A two-way Analysis of Variance (ANOVA) test revealed that Ach produced decreased vasodilation in aortas of MnD and MnS rats, in comparison to MnA. The vessel sensitivity (lower pD_2) of the MnS aortas to Ach was reduced compared to the MnA/ Control group, while MnD and MnA animals appeared to have similar vessel sensitivity to the agonist (Ach). The absence of dietary Mn appeared to reduce the

vasodilatory effect of the endothelium, possibly by affecting nitric oxide production or bioavailability. The above alterations in Ach-induced vasodilation could not be attributed to membrane-related effects. The presence of Mn in levels of supplementation (45-50 ppm) reduced Ach-induced vasodilation and vessel sensitivity to the agonist, acting as a non-competitive antagonist on the vasodilatory effect. The effect of the different dietary Mn concentrations on SNP-induced vasodilation was similar but much smaller when compared to Ach-induced vasodilation. Dietary Mn seems to affect vasodilation by acting primarily on the NO pathway and to a lesser extent on the cGMP pathway of the vascular smooth muscle. Our results confirm the effect of Mn on endothelium-dependent vasodilation in rat aorta and its multifunctional role on the regulation of vasomotor tone.

3.1. Introduction

The endothelium consists a physical barrier of the aortic tissue between circulating blood components and the perivascular tissues. One of the major roles of the endothelial layer is the regulation of vascular tone by releasing relaxing factors that serve as second messengers and regulate the biomechanical properties of the vessel [Fleisch, 1974]. These second messengers can be divided in two classes: the endothelium-derived relaxing factors (EDRFs) and the endothelium-derived contracting factors (EDCFs) [Luscher & Vanhoutte, 1990][Moncada et al, 1993][Moncada et al, 1991]. Nitric oxide (NO), prostacyclin (a product of the cyclooxygenase pathway) and endothelium-derived hyperpolarizing factor (EDHF) are among the major relaxing factors and cause dilation of the vascular smooth muscle. Endothelin-1, thromboxane and prostaglandin H_2 are among the major endothelium-derived contracting factors (EDCFs) [Luscher & Vanhoutte,

1990]. Shear stress as well as cell membrane receptors activated by hormones and autacoids stimulate the release of nitric oxide (NO) from endothelium by the formation of L-citrulline from L-arginine [Russo et al, 2002]. NO diffuses to the vascular smooth muscle (VSM) and induces vasodilation through the activation of soluble guanylate cyclase (sGC) and the formation of cGMP [Lucas et al, 2000]. Prostacyclin, a product of cyclooxygenase, leads to the formation of cAMP by activating adenylate cyclase and acts synergistically with NO inducing vasodilation [Luscher & Vanhoutte, 1990][Salvemini, 1996]. Finally, EDHF induces vasodilation by hyperpolarizing the VSM cell membrane with the opening of K^+ ion channels [Feletou et al, 2000].

Endothelial dysfunction and vascular dysregulation are observed in many cardiovascular diseases such as atherosclerosis, hypertension, heart failure, coronary heart disease and stroke [Cai et al, 2000][De Vriese, 2000][Loscalzo, 2000][Boulanger, 1999][Shimokawa, 1999][Mombouli et al, 1999]. The dysfunction of the endothelial layer leads to impaired bioavailability of NO and thus NO deficiency, which has been implicated in a variety of pathological conditions [Russo et al, 2002]. Limited NO bioavailability might be a result of decreased synthesis, impaired bioavailability of the bioactive NO or enhanced NO inactivation and production of its metabolites [Matsuoka, 2001]. As a result, the signal transduction processes in which NO is implicated as a second messenger are obstructed as well as the normal or protective physiological processes that they regulate.

One of the major functions of Mn lies in its role as an enzyme cofactor (Mn-activated enzymes) or as a structural part of enzyme molecules (Mn-containing enzymes) [Wedler, 1994]. Arginase, manganese superoxide dismutase (MnSOD) and guanylate

cyclase (GC) are some of the major representatives of the Mn-metalloenzymes with important roles in vascular cell pathways. Arginase catalyzes the formation of L-ornithine from L-arginine, which is the substrate for NO formation [Mori et al, 2000]. Manganese superoxide dismutase of mitochondrial origin provides one of the major antioxidant mechanisms by causing dismutation of superoxide ($O_2^{-\bullet}$) leading to the formation of the less active form of hydrogen peroxide (H_2O_2) [Zanetti et al, 2001]. Thus, MnSOD prevents the interaction of superoxide with NO which leads to inactivation of NO and formation of its by-products (peroxynitrite ONOO-, nitrite NO_2 and nitrate (and NO_3). Inactivation of the EDNO will affect cell-signaling pathways of vasodilation.

In our previous studies we reported reduced contractility of rat intact aortic rings when challenged with an adrenergic agonist, observed when Mn was present at levels of supplementation (45-50ppm). However, the presence of dietary Mn in the absence of endothelium, appeared to increase vascular smooth muscle (VSM) contraction. Aortas of MnD animals presented increased vessel sensitivity (pD_2) to the adrenergic agonist in endothelium-intact aortic rings. This finding along with the increase in vascular force of contraction in MnS aortas in the VSM suggest that the effect of Mn on the regulation of vasomotor tone takes place through an endothelium-mediated pathway, possibly by affecting the vasodilator tone [Kalea et al, 2002][Kalea et al, 2003][Kalea et al, 2005]. There have been few *in vitro* [Kasten et al, 1994] and *in vivo* studies [Ensunsa et al, 2004], which have suggested different effects of Mn on endothelium-mediated vasodilation. The goal of this study was to investigate the effect of dietary Mn on Acetylcholine (Ach)-induced vasodilation (endothelium-dependent) and on Sodium

Nitroprusside (SNP)-induced vasodilation (endothelium-independent) in male Sprague-Dawley rats fed three different concentrations of Mn.

3. 2. Materials and Methods

3.2.1. Animal Model

Fourty eight weanling male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were randomly assigned to three groups of sixteen rats, each on a Mn deficient (MnD), Mn adequate (MnA) and Mn supplemented (MnS) diet with <1ppm, 10-15ppm and 45-50 ppm of Mn respectively. The above Mn levels were chosen because studies indicate that a Mn level of less than 1.0 ppm results in Mn deficiency in about thirteen to fifteen weeks [Taylor et al, 1997][Klimis-Tavantzis et al, 1993][Yang et al, 1998][Yang et al, 1998b][Kalea et al, 2005] and ten ppm dietary Mn levels have been reported by the American Institute of Nutrition (AIN) as an adequate level to prevent deficiency in the rat [Reeves et al, 1993]. The animals were individually housed in metal mesh-bottomed cages in an environmentally controlled room (22°C with a 12:12 hrs light:dark cycle). All animals were weighed weekly. The Animal Care and Use Committee of the University of Maine approved all animal care and experimental procedures.

3.2.2. Diets

Diets were mixed in our laboratory from purified ingredients [Yang et al, 1998a][Yang et al, 1998b][Kalea et al, 2005], they were prepared fresh and they were refrigerated at 4°C for maximum of three days after preparation. Diets contained dextrose

69.1%, egg white solids 20%, corn oil 6%, vitamin mix 1%, mineral mix 1.5%, biotin 0.2 ‰ and D-L-Methionine 0.4%. Vitamin (A.O.A.C. Special Vitamin Mixture, Harlan Tekland) and mineral mixes (MMP Biochemicals) were commercially prepared. Manganese content of the diets and tap water were determined 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 ppm). Diet content of Mn was tested every time following preparation. Tap water (below the detection limit for Mn) and food were provided *ad libitum*.

3.2.3. Tissue Sampling

At the end of the feeding period, rat food was withheld for 12-14 hrs. The animals were anesthetized in a chamber with 95% CO_2 / 5% O_2, for approximately 2 min. Thoracic aortas and livers were removed carefully and washed with physiologic salt solution (PSS, with composition in mmol/L: NaCl, 118, KCl, 4.7, $NaHCO_3$, 25, KH_2PO_4, 1.18, $MgSO_4$, 1.17, Dextrose, 11, $CaCl_2$, 1.25). Liver tissues were weighted, lyophilized, pulverized and analyzed for Mn content 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.

3.2.4. Vascular Ring Studies

3.2.4.1. Aortic Preparations

The thoracic aorta was excised and submerged in a petri dish filled with physiologic salt solution (PSS) at room temperature. The aortic segment was cleaned of adherent extravascular tissue and divided into four rings of 3 mm length, which were selected from the middle section of the vessel. One ring from each animal was used for the following protocols. Two stainless steel wire triangles (0.012 in diameter) were passed carefully through each ring, which was mounted in a 20 ml Radnotti tissue bath containing PSS (37°C, continuously bubbled with a 95% O_2 and 5% CO_2 mixture to keep the pH at 7.4). The aortic ring was attached through the one triangle to a fixed glass hook in the tissue bath and through the other triangle and a weightless wire hook to a force transducer for the measurement of isometric force. The transducer was connected to a tissue force analyzer (Model 410, MicroMed, Louisville, KY), which was continuously displayed and recorded on-line on a computer.

Each ring was stretched to produce a resting tension (preload) of 1.5g from the initiation of the experiment [Beach et al, 2001][Adeagbo et al, 1993][Kawabe et al, 2003]. Tissues were allowed to equilibrate for 45min. During this time, all rings were washed with PSS (37°C, pH 7.4) and were precontracted for 10min with one maximal dose of L-Phe (10^{-8} M) and one dose of Ach (10^{-8} M) to saturate the non-selective receptor binding sites for the agonists. After washing the tissues four times, the preload was corrected to the original baseline levels and remained unchanged throughout the experiment.

3.2.4.2. Endothelium-Dependent Vasodilation

Following preconditioning the aortic rings were precontracted with one maximal dose of the α_1 adrenergic agonist L-Phenylephrine (10^{-6}M) for 10min, which was the duration necessary for the contraction curve to reach a plateau. Following the L-Phe precontraction, cumulative applications of seven concentrations of Ach (3×10^{-9}, 10^{-8}, 3×10^{-8}, 10^{-7}, 3×10^{-7}, 10^{-6}, 3×10^{-6}M) were applied, similarly to previous protocols [Schuschke et al, 1992][Beach et al, 2001]. A drug-tissue contact time of six min was allowed for each Ach concentration to achieve the maximum dilation to the initial precontraction.

3.2.4.3. Endothelium-Independent Vasodilation

Following the above protocol (3.2.4.2), the aortic rings were washed four times over a 20min period with aerated PSS (37°C, pH 7.4) and were allowed to equilibrate to baseline to bring aortic tension slightly below or down to the initial preload level. Endothelium independent dilation was then assessed with seven doses of SNP (3×10^{-9}, 10^{-8}, 3×10^{-8}, 10^{-7}, 3×10^{-7}, 10^{-6}, 3×10^{-6}M) as previously described [Kawabe et al, 2003]. Sodium nitroprusside is a nitric oxide donor and a cGMP-dependent vasodilator. A drug-tissue contact time of four min was allowed for each SNP concentration to achieve the maximum dilation for each dose. One dose of the non-selective and endothelium-independent vasodilator papaverine (10^{-3} M) was used in the end to obtain maximal vasodilation. Papaverine is a non-specific inhibitor of phosphodiesterase in the smooth muscle; it increases cAMP and cGMP concentrations, inducing indirectly vasodilation.

3.2.5. Drugs

Acetylcholine chloride, Sodium Nitroprusside, Papaverine, L-Phenylephrine and salts for the stock solutions of the physiologic salt solution (PSS: NaCl, KCl, $NaHCO_3$, KH_2PO_4, $MgSO_4$, Dextrose, $CaCl_2$) were purchased in pure forms from Sigma-Aldrich Chemical Co (St Louis, MO, U.S.A.) and were dissolved in distilled water.

3.2.6. Statistical Analysis

Animal weights and hepatic Mn content were compared using a one-way analysis of variance (ANOVA). The relaxant effect to each Ach dose was expressed as a percentage dilation to the initial L-Phe precontraction. Concentration response curves were fitted by nonlinear regression with simple algorithm and EC_{50} and pD_2 (pD_2=-$\log_{10}EC_{50}$) were calculated. The pD_2 values for the rings of all animals show differences in the vessel sensitivity to the Ach receptor. All results were expressed as mean values ± standard error of the mean (SEM). The percentages of dilation and pD_2 values to Ach were compared in different two-way analysis of variance (ANOVA) tests in order to determine the effect of different diets on vasodilation and on vessel sensitivity. Student Newman-Keuls tests were used for statistical analysis and a p-value level of 0.05 or less was considered as statistically significant. The Sigmastat Statistical Program Package (SAS Institute, Cary, NC) was used to conduct the statistical analysis.

3.3. Results

3.3.1. Animal Growth

All animals fed a MnD, MnA or MnS diet gained weight. However, mean body weights were significantly different among all groups (Table 3.1). The difference in growth rate was statistically significant from the fourth week on as has been described before [Kalea et al, 2005][Yang et al, 1998a]. The differences in liver weights among diet groups (Table 3.1) were proportionate to the differences in body weight as appears in the similar percentages of liver weight to body weight of the rats (Table 3.1), an indicator of normal growth. Manganese deficiency was confirmed by hepatic Mn content, which was decreased only in the MnD group (Figure 3.1).

Table 3.1. Effects of dietary Mn on body weight, liver weight and hepatic Mn content.

Diet Groups	Body weight (g)	Liver weight (g)	Liver weight/ Body weight	Hepatic Mn content (ppm)
MnD	462 ± 13 *	13.57 ± 0.58*	2.9 ± 0.001	1.223 ± 0.05*
MnA	526 ± 22	16.09 ± 0.93	3.0 ± 0.001	5.483 ± 0.186
MnS	539 ± 6 *	16.69 ± 0.64*	3.09 ± 0.001	5.652 ± 0.243

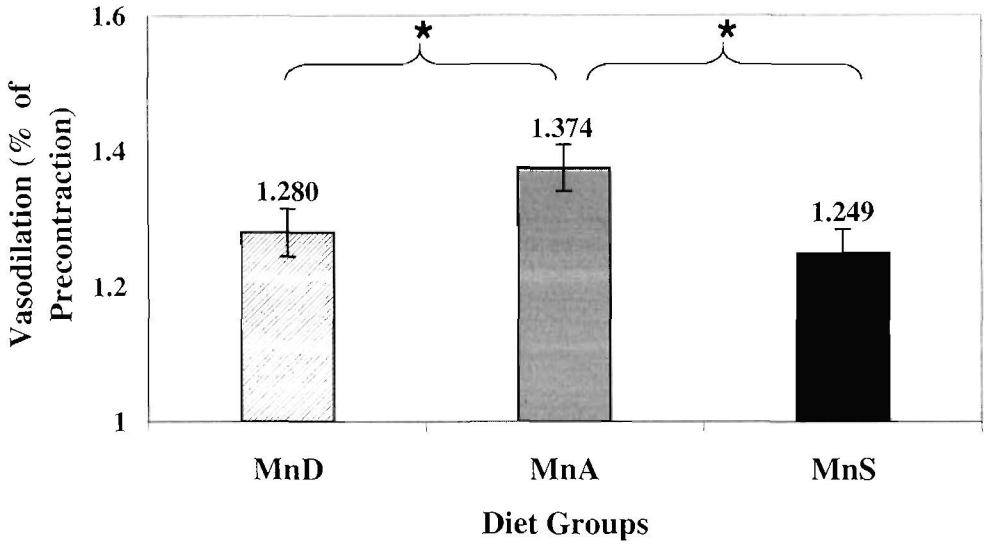
(*) Statistically significant differences at $p \leq 0.05$ compared to MnA/ Control group.

3.3.2. Endothelium-Dependent Vasodilation

Seven cumulative doses were used to give Ach concentrations of 3 x 10^{-9} to 3 x 10^{-6} M in three fold steps in the tissue baths. Each dose produced a graded decrease of the precontraction force that was obtained with one dose of L-Phe. A maximum contraction force was developed in response to the initial precontraction dose of L-Phe and was considered to be the point of 0% dilation (percentage of the level of precontraction), in order to set a baseline of initial precontraction similar for all rings. In 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, 0g of force developed) was considered to be the 100% dilation. Washout of agonists four times after the end of the protocol allowed the force to return at least to the preload value. All rings relaxed more than 100% in the completion of the Ach dose response curve. Acetylcholine-induced vasodilation was lower in both MnD and MnS diet groups when compared to MnA (Appendix, Figure A.1). Maximum % of vasodilation was significantly reduced in MnD and MnS groups when compared to MnA (Figure 3.1).

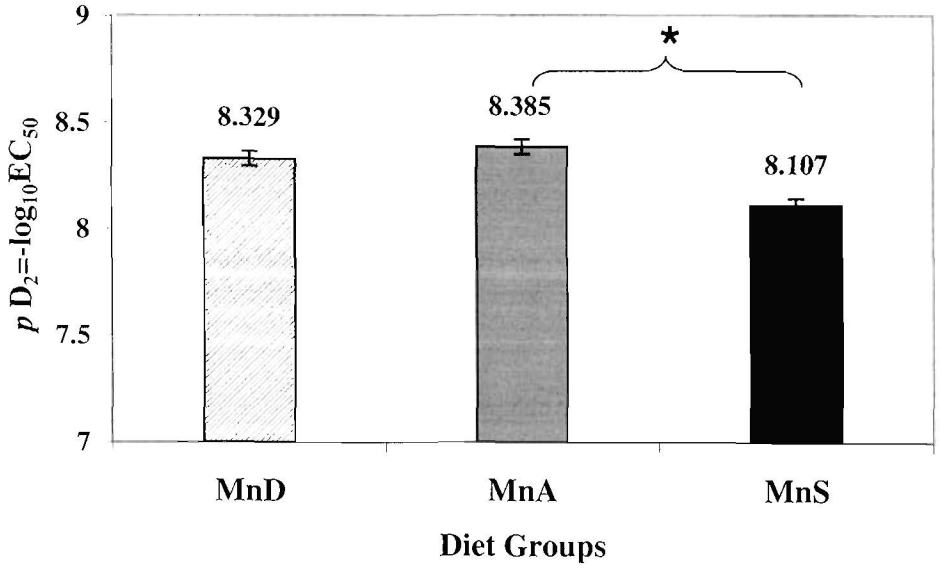
In order to determine the EC_{50} value, the mean maximum dilation force for each ring was used to calculate a percent decrease of force in each dose (the effective concentration of Ach in which 50 % of maximum dilation was obtained). The negative log (base 10) of the EC_{50} value was calculated to give us the pD_2 value for each ring, a measure of vessel sensitivity to the Ach receptor response.

Figure 3.1. Effects of dietary Mn on endothelium-dependent vasodilation (% of precontraction) of intact aortic rings in Sprague Dawley rat.



(*) Statistically significant differences at $p \leq 0.001$.

Figure 3.2. Effects of dietary Mn on vascular sensitivity to Ach (pD_2, mean value ± SEM) of aortic rings in Sprague Dawley rat.



(*) Statistically significant differences at $p \leq 0.001$.

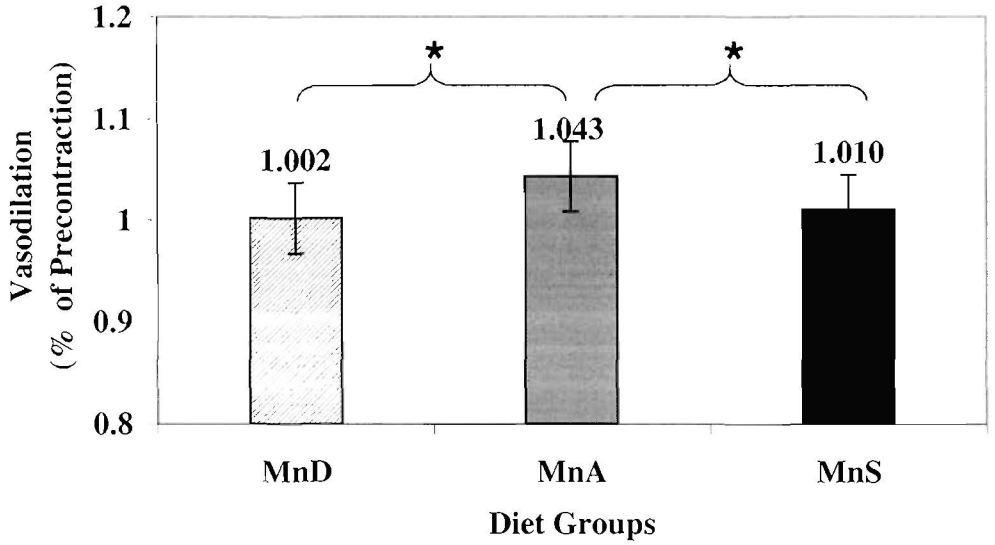
The pD_2 values for aortic ring dilations to Ach were compared among diet groups (Figure 3.2). Animals fed a MnD (<1 ppm) or a MnA (10-15 ppm) diet presented similar pD_2 values for Ach-induced relaxation of the L-Phe precontracted aortic rings. However, in animals fed a MnS diet (45-50 ppm) we observed a significantly reduced pD_2 value for Ach-induced relaxation when compared to MnA-fed animals (Figure 3.2). The relaxation to the one dose of papaverine was similar among diet groups.

3.3.3. Endothelium-Independent Vasodilation

Similarly, eight cumulative doses of SNP were used to give concentrations of 3 x 10^{-9} to 10^{-5} M in three fold steps in the tissue baths in order to decrease the precontraction force that was developed with the same dose of L-Phe. The decrease of force to the preload level (100% of dilation) was used to calculate a percent decrease in tension for each dose in each respective concentration-response curve to SNP. EC_{50} values were determined for each ring (the effective concentration of SNP in which 50% of maximum dilation was obtained). The negative log (base 10) of the EC_{50} value was calculated to give us the pD_2 value for each ring, a measure of vessel sensitivity to the SNP-induced dilation. The pD_2 values for aortic ring dilations to SNP were compared among diet groups.

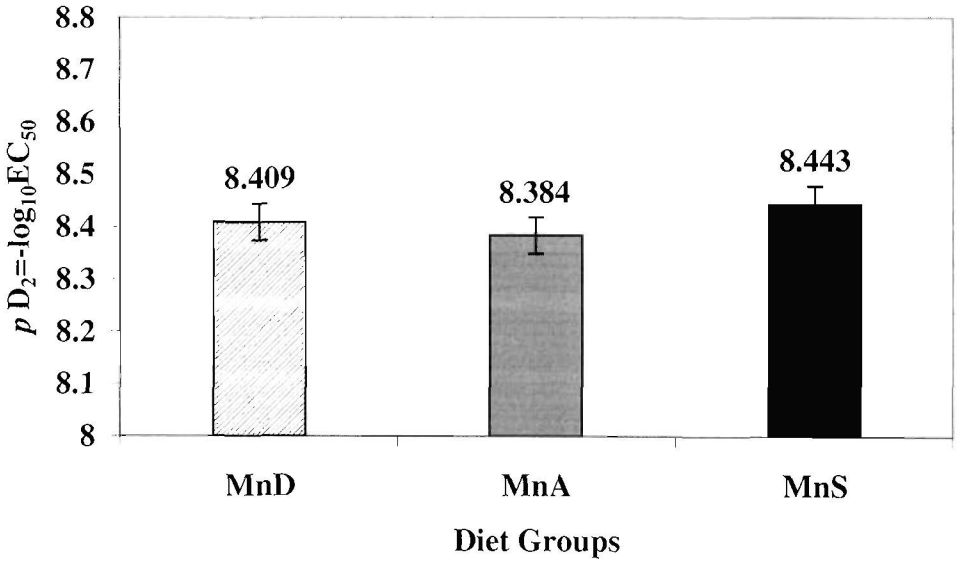
Even though we observed a reduced vasodilation to SNP in MnD and MnS groups, in comparison to MnA (Figure 3.3. and Appendix Figure A.2.), there were no statistically significant differences in vessel sensitivity to SNP among diet groups (Figure 3.4).

Figure 3.3. Effects of dietary Mn on endothelium-independent vasodilation (Fmax, mean value ± SEM) of aortic rings in Sprague Dawley rat.



(*) Statistically significant differences at $p \leq 0.001$.

Figure 3.4. Effects of dietary Mn on vascular sensitivity to SNP (pD_2, mean value ± SEM) of aortic rings in Sprague Dawley rat.



No statistically significant differences ($p>0.05$).

3.4. Discussion

In the present study we document that the presence of Mn in the diet affects primarily the function of the endothelial layer since we observed significant differences among diet groups in endothelium-dependent vasodilation (induced by Ach) and in a much smaller extend in endothelium-independent vasodilation as induced by a direct smooth muscle relaxant (induced by SNP) (Appendix, Figures A.1. and A.2). In MnS diet group we observed a pronounced decrease of the Ach receptor sensitivity (pD_2) compared to the MnA group (Figure 3.2). The vessel sensitivity (pD_2) to endothelium-independent vasodilation was not affected by the absence of Mn from the diet (<1ppm) neither its supplementation at levels of 45-50 ppm (Figure 3.4).

In Ach-induced vasodilation absence of Mn from the diet did not appear to affect the vessel sensitivity to the agonist (Ach), while it reduced the maximum dilation when compared to MnA group (Figures 3.1 and 3.2). Such an effect might be attributed to a reduction of the vasodilatory effect of the endothelial cell. Thus, inadequate Mn intake seems to reduce the production, release or bioactivity of endothelium-generated vasodilators, without affecting membrane-related events. We know that Mn is a cofactor for MnSOD, one of the major antioxidant defense systems of endothelial cells that scavenge the deleterious free radical superoxide. In severe Mn deficiency, the activity of MnSOD is reduced allowing the formation of superoxide [Malecki et al, 1994][Malecki et al, 1996], which reacts with and inactivates NO. An increase in formation of NO by-products is an indication of NO inactivation. Even though we have information on the reduced activity of heart, liver and kidney MnSOD in Mn deficiency, there is no information on the effect of Mn deficiency on MnSOD of the vascular tissue [Ensunsa et

al, 2004][Malecki et al, 1994][Malecki et al, 1996]. Similarly, reports on the increased formation of NO by-products are limited and concern only enzyme activity in liver tissue [Ensunsa et al, 2003].

When Mn is present in levels of supplementation (MnS) the vessel sensitivity to Ach is decreased, suggesting that dietary intake of Mn at 45-50 ppm might affect the receptor-agonist interaction. Activation of the Ach receptor in the vascular endothelium results in an influx of Ca^{+2} and stimulation of NOS III, which leads to the production of NO from L-arginine. Nitric oxide diffuses to nearby smooth muscle cells and reacts with the ferrous iron in the heme prosthetic group of the sGS. Activation of sGC catalyzes the formation of cGMP from GTP, which leads to dephosphorylation of myosin light chains in the contractile machinery and to vasodilation [Moncada et al, 1993]. Elevated production of NO through the activity of NOS seems to be responsible for vascular hypocontractility. Additionally, Ach leads to the production of endothelium-derived vasodilators such as eicosanoids, which contribute to endothelium-dependent vasodilation [Vanhoutte, 1990]. Hyperpolarization of the VSM cell membrane by Ach contributes the diffusible EDHF, which induces vasodilation and is different from NO and prostacyclin [Vanhoutte, 2003]. Potassium ions, hydrogen peroxide and epoxyeicosatrienoic acid are among the most recent speculations on the nature of EDHF, which still remains unknown [Félétou et al, 2000]. In MnS diet, a decrease in Ach-induced vasorelaxation could be due to an enhanced Phe-induced precontraction of the VSM, which has been reported previously [Kalea et al, 2003]. It might also suggest a decrease in synthesis/ release of NO from endothelial cells due to increased degradation of NO or an effect of dietary Mn on the Ach receptor.

It has been well documented in the past that dietary Mn affects the concentration and synthesis of proteoglycan and glycosaminoglycan (GAG) structures in rat aorta [Yang et al, 1998][Yang et al, 1998b][Klimis-Tavantzis et al, 1989]. We have also recently reported alterations in the disaccharide composition and sulfation pattern of heparan sulfate glycosaminoglycans [Kalea et al, 2005]. The highly diverse structure of GAG molecules enables them to interact in divergent ways with biologically effective molecules, such as receptors, enzymes, cytokines, growth factors and proteins [Wight et al, 1992]. Oversulfated disaccharide domains facilitate the binding of HSPGs with growth factors, matrix components, effectors and modulators of enzymatic catalysis [Militsopoulou et al, 2003], since sulfonation is the recognized primary mechanism that influences biological activity by determining high specificity in these receptor-ligand binding interactions [Strott, 2002][Lyon et al, 1998].

Even though the physiological role of HSPGs as co-receptors is still not clear in many cases, it seems that overexpression of HSPGs increases vascular sensitivity to agonists, an indicator of the binding affinity of the ligand with the specific receptor, especially in low ligand concentrations [Risler et al, 2002][Kramer KL et al, 2003]. It has also been reported that oversulfated HS proteoglycans upregulate the gene expression and clustering (aggregation) of Ach receptors in vitro [Meier et al, 1998][Jones et al, 1996][McDonnel et al, 2004]. In our recent studies, aortas from MnS-fed rats (45-50 ppm) presented a significantly higher concentration of non-sulfated disaccharides, while MnD and MnA appeared to have increased concentration of oversulfated (tri- and di-) HS disaccharides. A reduced sulfation in the HS proteoglycans on the MnS rats might affect the expression or activation of Ach receptors in this diet group, in which we could

attribute the decreased vessel sensitivity to Ach in MnS animals, provided that we do have maximum receptor occupancy in our experimental conditions. However, in our study we did not measure directly the receptor number, affinity or occupancy in our experiments, which would be necessary to support such hypothesis.

Ensunsa et al. [Ensunsa et al, 2004] recently reported a decreased vascular sensitivity to Ach in aortic rings from rats fed a MnS diet, while they did not observe any statistically significant differences to SNP-induced vasodilation. In their experimental protocol they have used MnD female offsprings of MnD Sprague Dawley rats and compared them with MnS-fed rats (45 ppm) without using a MnA/ Control diet group. Our results are in accordance with the findings of this study (reduced Fmax and pD_2 in MnS group when compared to MnD). The same study [Ensunsa et al, 2004] supported that the increased vasodilation observed in the MnD diet group (compared to MnS) may be attributed to the increased availability of NOS and NO production due to the attenuated activity of arginase (which competes with eNOS for the substrate L-arginine) in the MnD diet group. However, this and previous studies reported a reduced liver and kidney but not vascular arginase activity in MnD diets. Arginase levels have always been compared to diets with Mn concentration much higher than 10-15 ppm (45, 48 and 50ppm) [Brock et al, 1994][Kirchgessner et al, 1978][Sabbatini et al, 2003][Ensunsa et al, 2004]. Thus, we can not support that arginase deficiency in vascular tissue might affect the endothelium-dependent vasodilation in our experiments.

The reduced Ach-induced vasodilation in MnD and MnS diet groups might reflect a change in the sensitivity of the vascular smooth muscle to relaxation by NO. Such an effect though would alter the responses to the NO-donor SNP, which is not supported

from our observations on the endothelium-independent vasodilation. Sodium nitroprusside is a NO-donor, which acts on cGMP to induce vasorelaxation. Even though Mn is a cofactor for sGC which catalyzes the formation of cGMP, the effect of Mn on VSM vasodilation seems to be of limited biological significance in comparison to its effect on endothelium-mediated vasodilation. Additionally, vessel sensitivity to the NO donor (pD_2 for SNP) was similar among diet groups. Our data suggest that dietary Mn does not have a pronounced effect on vasodilation at post-receptor level.

Our results suggest that the presence of Mn in the diet in concentrations lower or higher than 10-15ppm (adequate levels) reduces endothelium-dependent vasodilation by different mechanisms. In Mn deficiency the vasodilatory effect of the endothelium is reduced, possibly due to a compromised antioxidant defense system which reduces NO bioavailability. In levels of supplementation (45-50 ppm) the effect of Mn might be attributed to an enhanced VSM contraction or an effect on the Ach receptor expression or activation. The mechanisms responsible for the alterations on the endothelium-dependent vasodilation observed in the MnS diet group may include possible alterations in the L-arginine/NO pathway (generation of NO) or in the cyclooxygenase-dependent EDCF production which interacts with the NO pathway and need to be further dissected.

Chapter 4

The Effect of Dietary Manganese on Nitric Oxide Synthesis in Rat Aorta

Previously we reported that dietary manganese (Mn) affects the endothelium-dependent vasodilation pathways in rat aorta and has a smaller effect on cGMP pathway in the vascular smooth muscle (VSM). In the present study we examined the mechanism by which dietary Mn might affect two different endothelium-dependent vasodilation pathways, the L-arginine/nitric oxide (NO) and the cyclooxygenase (COX) pathway. Weanling male Sprague-Dawley rats were fed either a Mn deficient (MnD), adequate (MnA/ Control) or supplemented (MnS) diet (<1, 10-15 and 45-50 ppm Mn respectively). Following 14 weeks on the diet rats were sacrificed and four 3 mm aortic rings were prepared. One ring from each animal was incubated with physiological salt solution (PSS) only. Two of the three aortic rings from each animal were suspended in PSS with inhibitors: one ring with the inhibitor of nitric oxide synthase (NOS) L-N^G-monomethyl-arginine (L-NMMA, 10^{-4} M) and one ring with the COX inhibitor mefenamic acid (MFA, 10^{-5} M). The third ring was incubated in PSS with L-arginine (10^{-3} M), the substrate for NO formation. All rings were equilibrated and preconditioned for 45 min and they were precontracted with one dose of the α_1 adrenergic receptor agonist L-Phenylephrine (L-Phe, 10^{-6} M) to induce maximum contraction. Cumulative applications of seven concentrations of Acetylcholine (Ach, 3×10^{-9}, 10^{-8}, 3×10^{-8}, 10^{-7}, 3×10^{-7}, 10^{-6}, 3×10^{-6} M) were applied to induce endothelium-dependent vasodilation. The vasodilatory effect to each Ach dose was expressed as a percentage relaxation to the initial L-Phe precontraction. The maximal % of vasodilation was compared among rings from different

diet groups. The percentage of relaxation to the initial L-Phe dose of precontraction was used to determine the vessel sensitivity (pD_2) to Ach. Enzyme expressions for NOS II and III were determined with western blot analyses. MnD diet resulted in depressed animal growth and lower hepatic Mn content. Expressions of NOS II and III were similar among diet groups. The addition of L-arginine did not increase NO-mediated vasodilation in any of the diet groups. A two-way Analysis of Variance (ANOVA) test revealed that the differences in the reduction of vasodilation induced by NOS inhibition with L-NMMA were similar among diet groups. Thus, dietary Mn does not affect the availability of L-arginine for NO synthesis and has no effect on the expression of NOS II and III isoforms. In the presence of a COX inhibitor, we observed an enhanced vasodilation of MnD rings, which suggests the presence of an endothelium-derived vasoconstrictor in the MnD group. Our results indicate that Mn affects endothelium-mediated vasodilation by preserving NO bioavailability and the absence of Mn from the diet might cause the release of an endothelium-derived vasoconstrictor.

4.1. Introduction

Endothelial dysfunction is defined by an impairment of vasodilator response to the flow of agonists, which involves multiple interacting signaling pathways. A decrease in the bioavailability of endothelium-derived vasodilators such as nitric oxide (NO) seems to be one of the primary risk factors in the development of several vascular diseases. Nitric oxide is synthesized by endothelial cells and has an important regulatory role in the cardiovascular system. Activation of specific receptors in the endothelial cell membrane by agonists such as Acetylcholine (Ach) results in an increase in intracellular

calcium which activates nitric oxide synthase (NOS) (Figures 1.2., 1.3. and 1.4.). L-Arginine is the physiological precursor for NO formation which requires NOS, as well as cofactors such as tetrahydrobiopterin (BH_4), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), molecular oxygen and NADPH [Moncada & Higgs, 1993]. There are three isoforms of NOS, the neuronal NOS (nNOS or NOS I), endothelial NOS (eNOS or NOS III) (both NOS I and III are constitutively expressed at low levels in a variety of cell types) and the inducible NOS (iNOS or NOS II) which normally is not expressed in most cell types and is induced by bacterial endotoxin and inflammatory cytokines [Wu & Morris, 1998]. Reduced NO bioavailability is the result of a decreased production (limited substrate availability, impaired NOS activity or expression) or increased degradation or accelerated scavenging of NO [Arnal et al, 1999]. Under oxidative stress, an interaction of superoxide with NO leads to the destruction of its vasodilatory actions and the production of cytotoxic NO by-products such as peroxynitrite ($ONOO^-$). This process is related not only to reduced vasodilation but also to pathologic conditions such as hypertension [John et al, 2000], atherosclerosis [Russo et al, 2002] and diabetes [De Vriese, 2000].

Nitric oxide has also been shown to interfere with endothelial cell-arachidonic acid (AA) pathways by stimulating the activity of cyclooxygenase (COX) in the production of eicosanoids [Salvemini, 1996][Soma et al, 1996][Wang et al, 1997]. Eicosanoids are products of the AA metabolism with significant biological role in vascular functions. They comprise a group of endogenously produced vasoactive mediators with diverse roles in the regulation of vasomotor tone. Nitric oxide seems to regulate the synthesis of vasodilators such as prostaglandin H (PGH) and prostaglandin I_2

(PGI_2) synthase, as well as vasoconstrictors such as thromboxane A_2 (TXA_2). Alterations in the ratio between the vasoconstrictor and vasodilator eicosanoids are specifically important for the development of vascular dysfunction. Nitric oxide provides permanent negative feedback and thus, restricts the release of endothelium-derived vasoconstrictors, a function influenced by oxidative stress. The presence of reactive oxygen species has been shown to favor the production of vasoconstrictor (TXA_2) instead of vasodilator prostanoids (PGI_2) [Laemmel et al, 2003]. There is limited information on the effects of trace minerals and more specifically Mn, on eicosanoid metabolism in the vascular environment, as related to vascular function.

Dietary manganese (Mn) has been reported to affect the vasoconstriction pathways by antagonizing Ca^{+2} ions in the endothelium, thus impairing Phe-induced contractions in intact rings and potentiating VSM contractions. The presence of Mn seems to impair the endothelial function rather than the vascular responsiveness to the endothelium-derived vasodilator nitric oxide (NO) (Chapter 3). The impairment in endothelium-dependent vasodilation is observed in dietary concentrations lower (Mn <1 ppm) or higher (Mn 45-50 ppm) than the recommended dietary intake (MnA diet, Mn 10-15 ppm) that is suggested by the American Institute of Nutrition [Reeves et al, 1993].

The purpose of the present study was to determine the effect of Mn on the vascular ability to synthesize NO by determining:

a. the ability of utilizing L-arginine, the substrate for NO synthesis in our diet (L-arginine was added directly in the tissue bath to restore any reduction in dilation response that could be caused by a reduced availability of the substrate),

b. the effect of NOS inhibition on vasodilation among diet groups by using an L-arginine analogue L-NMMA (L-N^G-monomethyl arginine). L-NMMA inhibits NO synthesis in a dose-dependent specific manner by inducing a small but significant endothelium-dependent contraction and inhibits the release of NO induced by Acetylcholine (Ach) [Moncada et al, 1989],

c. the expression of the two isoforms of nitric oxide synthase (NOS), the endothelial NOS (eNOS or NOS III) and the inducible NOS (iNOS or NOS II),

d. the importance of the COX pathway in mediating Ach-induced vasodilation among diet groups, by inhibiting the activity of COX I and II with mefenamic acid (MFA).

4. 2. Materials and Methods

4.2.1. Animal Model

Aortas from the thirty weanling male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were randomly assigned to a Mn deficient (MnD), Mn adequate (MnA) and Mn supplemented (MnS) diet (<1ppm, 10-15ppm and 45-50 ppm of Mn respectively by analysis). The animals were individually housed in stainless steel cages in an environmentally controlled room (22°C with a 12:12 hrs light:dark cycle). Body weight was measured weekly. The Animal Care and Use Committee of the University of Maine approved all animal care and experimental procedures.

4.2.2. Diets

Diets were mixed in our laboratory from purified ingredients, as described before [24, 25]. Vitamin (A.O.A.C. Special Vitamin Mixture, Harlan Tekland) and mineral mixes (ICN Biochemicals, Cleveland, OH) were commercially prepared. Manganese content of tap water and of the diets was determined 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 ppm). The rats were given free access to water (tap water, below the detection limit for Mn) and to one of the three diets for 14 weeks.

4.2.3. Vascular Ring Studies

At the end of the feeding period, rat food was withheld for 12-14 hrs. Animals were anesthetized in a chamber with 95% CO_2 / 5% O_2, for approximately 2 min. Thoracic aortas were excised and washed with PSS, with composition in mmol/L: NaCl, 118, KCl, 4.7, $NaHCO_3$, 25, KH_2PO_4, 1.18, $MgSO_4$, 1.17, Dextrose, 11, $CaCl_2$, 1.25.

4.2.3.1. Aortic Preparations

The thoracic aorta was removed carefully and submerged in a petri dish filled with physiologic salt solution (PSS) at room temperature. The aortic segment was cleaned of fat and connective tissue and divided into four rings of three mm length prepared from the middle section of the vessel. Each ring from each animal was used for one of the four different treatments. Two stainless steel wire triangles (0.012 in diameter)

were passed carefully through each ring. Each aortic ring was mounted in a 20ml Radnotti tissue bath, which contained PSS maintained at 37°C by a thermoregulated water circuit and was continuously bubbled with a 95% O_2 and 5% CO_2 mixture in order to keep the pH at 7.4. The aortic ring was attached through the one triangle to a fixed glass hook in the tissue bath and through the other triangle and a weightless wire hook to a force transducer. The transducer was connected to a tissue force analyzer (Model 410, MicroMed, Louisville, KY) for the measurement of isometric force, which was continuously displayed and recorded on-line on a computer.

4.2.3.2. Experimental Protocol

A total of 64 endothelium-intact rings were used for each of the four different protocols, sixteen rings from each diet group. Each ring was stretched to produce a resting tension (preload) of 1.5g from the initiation of the experiment [28-32]. Tissues were washed with PSS after the application of preload and then four different treatments were applied to the four different rings before the preconditioning with Ach and Phe and the equilibration.

One ring was washed with PSS without the addition of a substrate or an inhibitor. In a second ring we added PSS plus a NOS inhibitor (L-NMMA, 10^{-4}M, inhibitor of NOS I, II and III) into the bath for 10min, which remained in the tissue bath throughout the experiment. In a third ring a cyclo-oxygenase inhibitor (Mefenamic acid, 10^{-5} M, inhibitor of cyclo-oxygenase I and II) was added with PSS group for 10min and remained again in the tissue bath throughout the experiment. In a fourth ring L-arginine (10^{-3} M) was added in the tissue bath for 10min and added back after each wash out.

All rings were precontracted for 10min with one maximal dose of L-Phe (10^{-8} M) and one dose of Ach (10^{-8} M), to saturate the non-selective receptor binding sites for the agonists. After washing the tissues four times the inhibitors (L-NMMA and MFA) and the L-arginine were added back to the tissue baths, preload was corrected to the original baseline levels and remained unchanged throughout the experiment. All aortic preparations were left to equilibrate for approximately 45min before the application of the dose response curve.

Following preconditioning, all three aortic ring preparations were precontracted with one maximal dose of the α_1 adrenergic agonist L-Phe (10^{-6}M) for 10min, which was the duration necessary for the contraction curve to reach a plateau. Following the L-Phe precontraction, cumulative applications of seven concentrations of Ach (3×10^{-9}, 10^{-8}, 3×10^{-8}, 10^{-7}, 3×10^{-7}, 10^{-6}, 3×10^{-6}M) were applied. A drug-tissue contact time of six min was allowed for each Ach concentration to achieve the maximum relaxation to the initial precontraction.

4.2.4. Western Blot Analyses of NOS II and NOS III

Aortic segments were placed under liquid nitrogen and frozen at –80°C to be used for determination of NOS II and NOS III expression in each animal group. Frozen aortic segments (0.1gm dry weight) were powdered under liquid nitrogen and placed for 30min on three volumes of homogenization buffer on ice [50mM Tris-HCl, 5 mM EDTA, 10mM benzamidine, 10% glycerol, 50μg/ml phenylmethyl sulfonyl fluoride (PMSF), 10μg/ml aprotinin, 10μg/ml pepstatin A and 10μg/ml leupeptin (v/v) (pH 7.4-7.5)]. Powdered aortas were homogenized with a tissue homogenizer (Tissue Tearor, Biospec)

under 25rpm for 5min on ice. The homogenate was sonicated for 3min and placed on a rocking platform with 5% 3-[(3-chloramidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) for 2hrs at 4°C. Following a centrifugation at 12,000g for 30min, the supernatant was collected and total cellular protein concentration was determined by using the Bicinchoninic acid assay (BCA Protein Assay Kit, Pierce) with bovine serum albumin (BSA) as a standard.

Protein samples 100μg/ lane from each diet group were separated in SDS-PAGE electrophoresis, one for NOS II and one for NOS III determination. Human endothelial cell lysate (BD Transduction Laboratories) was used for a positive control for NOS III and mouse macrophage+IFNγ/LPS (BD Transduction Laboratories) for NOS II positive control. High range molecular weight markers (Bio-Rad) were separated by electrophoresis under reducing denaturing conditions in 7.5% polyacrylamide/ SDS gels (Bio-Rad) (150V for 45min) and transferred by electroblotting in onto PVDF blots (100V for 1hr and 15min at 4°C). Gels were stained with Coomasie blue stain to confirm complete protein transfer.

Blots were blocked for an hour in room temperature in blocking buffer of 5% nonfat dry milk (Bio-Rad) in TBST-20 on a rocking plate. They were then incubated with the primary antibody overnight in 4°C. The primary antibody for the NOS III was the anti-endothelial nitric oxide synthase (anti-NOS III) from BD Transduction Laboratories (1:1000 diluted in blocking buffer), while the primary antibody for NOS II was the anti-inducible nitric oxide synthase antibody (anti-NOS II) from the same company (1:1000 diluted in blocking buffer). Antibodies were diluted in 5% nonfat dry milk by adding 20μg of the antibody preparation in 20ml of the blocking buffer. The membranes were

washed 5 times with TBST-20 every 15 min and were then incubated with the same secondary antibody, which was a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:1000 diluted in blocking buffer, BD Transduction Laboratories) for 4hrs at room temperature. All membrane incubations were performed on a rocking platform. The membranes were washed as previously described and signal detection was facilitated after treatment with enhanced chemiluminescence reagents (ECL kit, Amersham). X-Ray films were developed for 1 min and densitometric analysis was realized on the scanned film images using Scion Image-Release Beta 4.02 software (http://www.scioncorp.com). Results were expressed as the percentage of protein staining vs. basal expression in positive control sample.

4.2.5. Drugs and Chemicals

Acetylcholine chloride, Sodium Nitroprusside, L-Phenylephrine, L-NMMA, L-Arginine, Mefenamic acid and salts for the stock solutions of the physiologic salt solution (PSS: NaCl, KCl, $NaHCO_3$, KH_2PO_4, $MgSO_4$, Dextrose, $CaCl_2$) were purchased in pure forms from Sigma-Aldrich Chemical Co (St Louis, MO, U.S.A.). Reagents for SDS-PAGE and Western blot were purchased from BioRad Laboratories, the BCA Protein assay kit from Pierce and the ECL Kit as well as the X-Ray ECL films from Amersham. Antibodies and positive controls were purchased from BD Transduction Laboratories.

4.2.6. Statistical Analysis

Animal weights and hepatic Mn content were compared using a one-way analysis of variance (ANOVA). The relaxant effect to each Ach dose was expressed as a percentage relaxation to the initial L-Phe precontraction. Concentration response curves were fitted by nonlinear regression and EC_{50} and pD_2 ($-\log_{10}EC_{50}$) were calculated. The pD_2 values indicate the vessel sensitivity to the Ach-receptor. All results were expressed as mean values ± standard error of the mean (SEM). The percentages of relaxation and pD_2 values to Ach were compared in different two-way analysis of variance (ANOVA) tests in order to determine the effect of different diets on vasodilation and on vessel sensitivity. Student Newman-Keuls tests were used for statistical analysis and a *p*-value level of 0.05 or less was considered as statistically significant. The statistical program used was the Sigmastat Statistical Program Package (SAS Institute, Cary, NC). Results from the densitometric analyses of the scanned western blot film images were expressed as the percentage of protein staining *vs.* basal expression of the positive controls.

4.3. Results

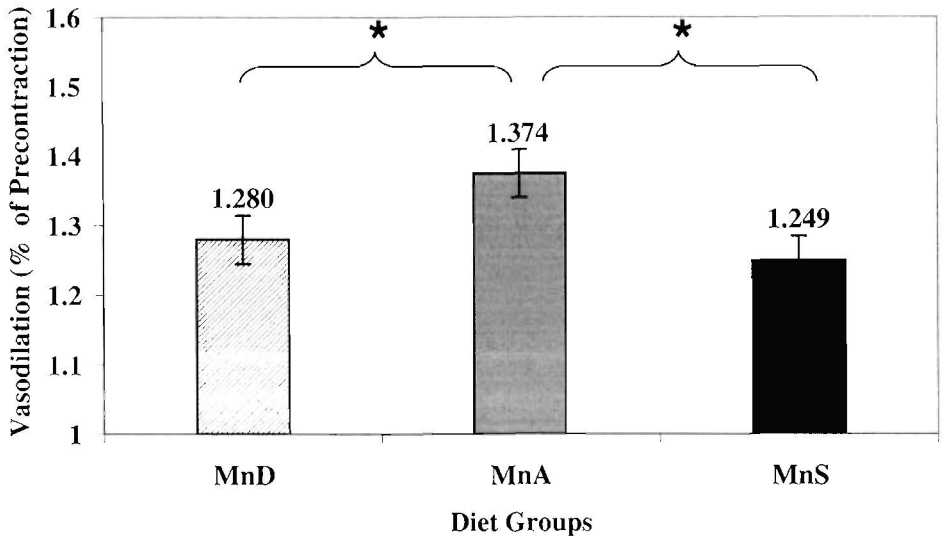
4.3.1. Animal Growth

Mean body weights of MnD animals on the 14^{th} week were significantly lower than MnA weights, while rats on MnA and MnS had similar weights (Table 4.1). Liver weights of the MnD rats were reduced when compared to MnA; a reduction proportionate to the total body weight reduction. Manganese deficiency was confirmed by hepatic and

kidney Mn concentration, which significantly decreased only in the MnD group (Refer to Table 3.1, pg.58).

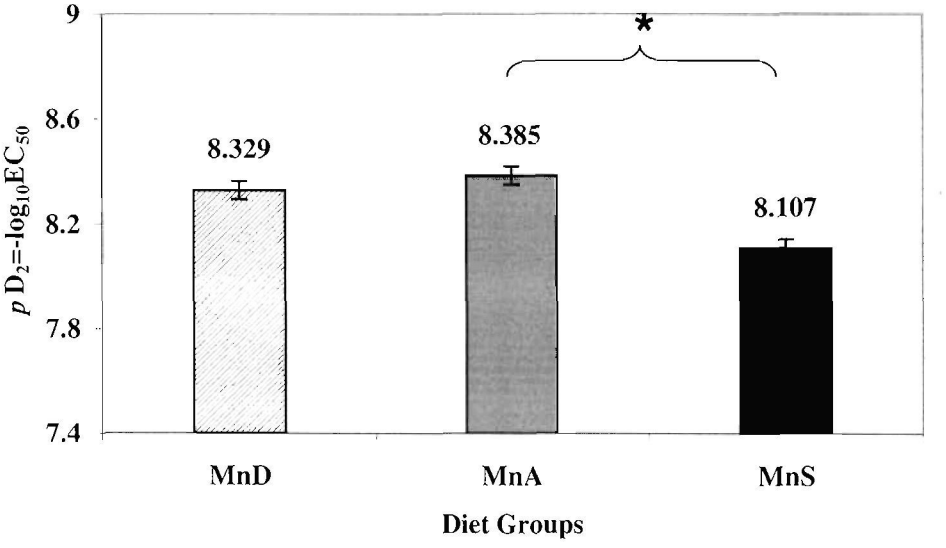
The effect of Mn on Ach-induced vasodilation (Figures 4.1, 4.2, Appendix Figures A.1 and A.2) is discussed in Chapter 3. Briefly, we observed a reduced vasodilation in MnD and MnS rings when compared to MnA rings and increased vessel sensitivity to Ach in MnS rings. Below we discuss the effect of dietary Mn on the enhancement or impairment of Ach-induced vasodilation by the presence of L-arginine or the NOS and COX inhibitors.

Figure 4.1. Effects of dietary Mn on Ach-induced vasodilation (expressed as % of Precontraction) of intact aortic rings in Sprague Dawley rat.



(*) Statistically significant differences at $p \leq 0.001$ in comparison to MnA/ Control group.

Figure 4.2. Effects of dietary Mn on vascular sensitivity to Ach (pD_2, mean value ± SEM) of aortic rings in Sprague Dawley rat.



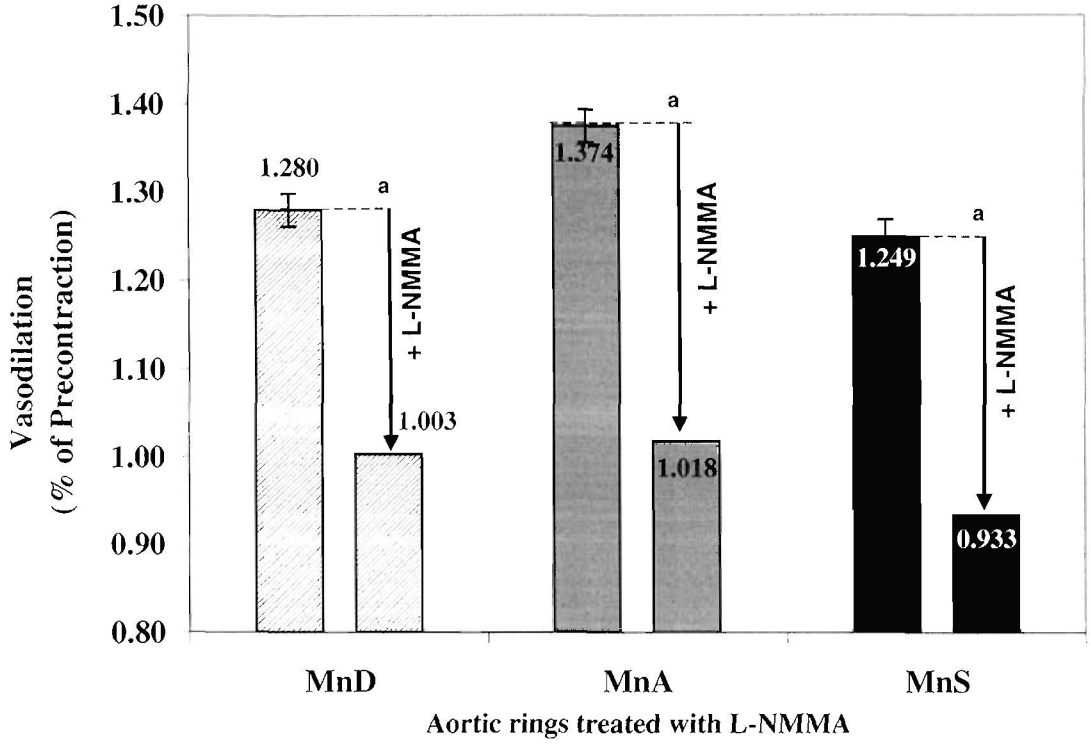
(*) Statistically significant differences at $p \leq 0.001$ in comparison to MnA/ Control group.

4.3.2. Effects of Dietary Mn on Vasodilation after NOSIinhibition

L-NMMA reduced the response to Ach in all diet groups (Figure 4.3 and A.3). Vasodilation of the L-NMMA treated aortas from MnS animals was significantly lower when compared to MnD and MnA groups. The differences in reduction of vasodilation after treatment with L-NMMA is depicted with arrows above the bars in Figures 4.3 and 4.4 and were not statistically different among diet groups (p=0.119) (as indicated by the same letters and same length of arrows in Figure 4.3). Thus, the reduction in vasodilation in MnS group when compared to Control (0.933 *vs.* 1.018), appears to be an effect of the reduced vasodilation in this group when the inhibitor is not present (1.249 *vs.* 1.374).

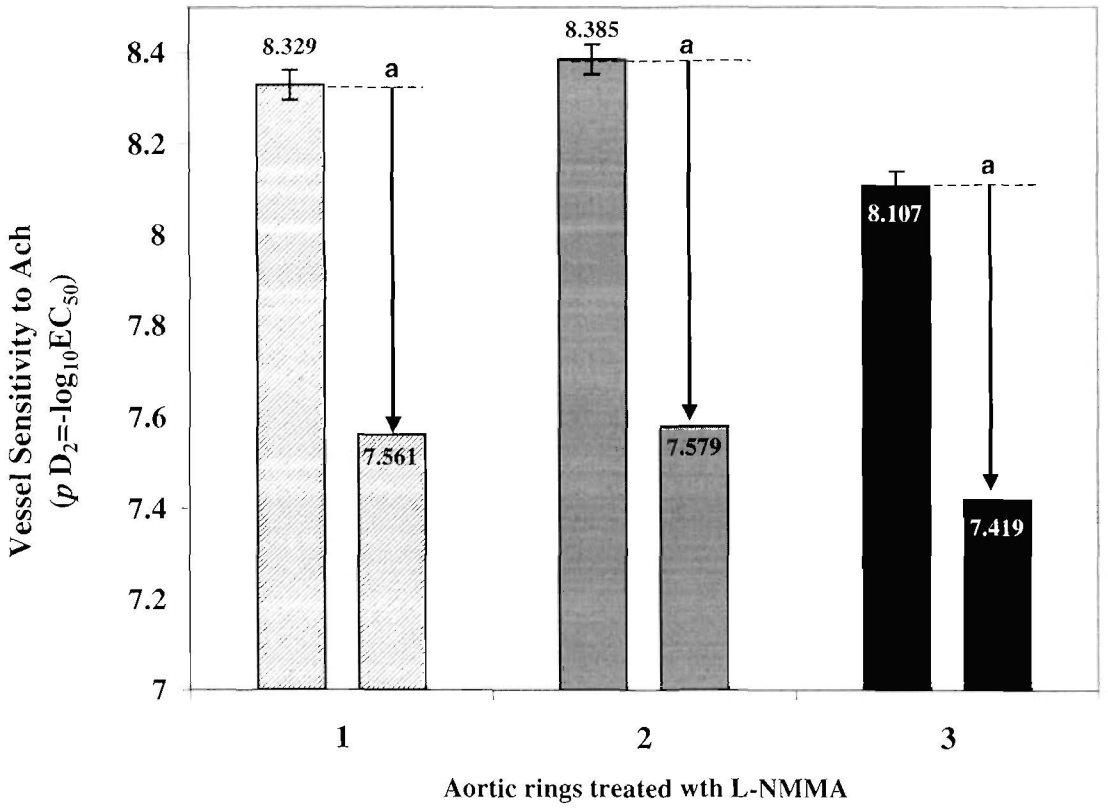
The vessel sensitivity to Ach in the presence of L-NMMA was reduced in all diet groups on the same level, as described by similar differences in L-NMMA-induced decreases of pD_2 values (Figure 4.4). The reduced vessel sensitivity to Ach in MnS diet group in the presence of a NOS inhibitor indicates that this alteration occurs due to the effect of Mn on the receptor-agonist relationship (in our case on Ach and its receptor) and not on the decrease in synthesis/ release of NO from endothelial cells. The effect of Mn on enhancing vasodilation seems to be independent from NOS enzymes. Reduced vessel sensitivity to Ach in all groups in the presence of L-NMMA (Figure 4.4) has been reported in the past and is an indicator of the increased need for bound Ach in order to reach the same NO effect.

Figure 4.3. Effects of dietary Mn on the maximum Ach-induced vasodilation (expressed as % of Precontraction) after treatment with L-NMMA in Sprague Dawley rat aorta.



(a, b) Different letters on the top of the arrows depict statistically significant differences on the length of the arrows, which symbolizes the reduction in Ach-induced vasodilation after the addition of the NOS inhibitor substrate (L-NMMA) in each individual diet group.

Figure 4.4. Effects of dietary Mn on the vascular sensitivity (pD_2) to Ach after treatment with L-NMMA in Sprague Dawley rat aorta.



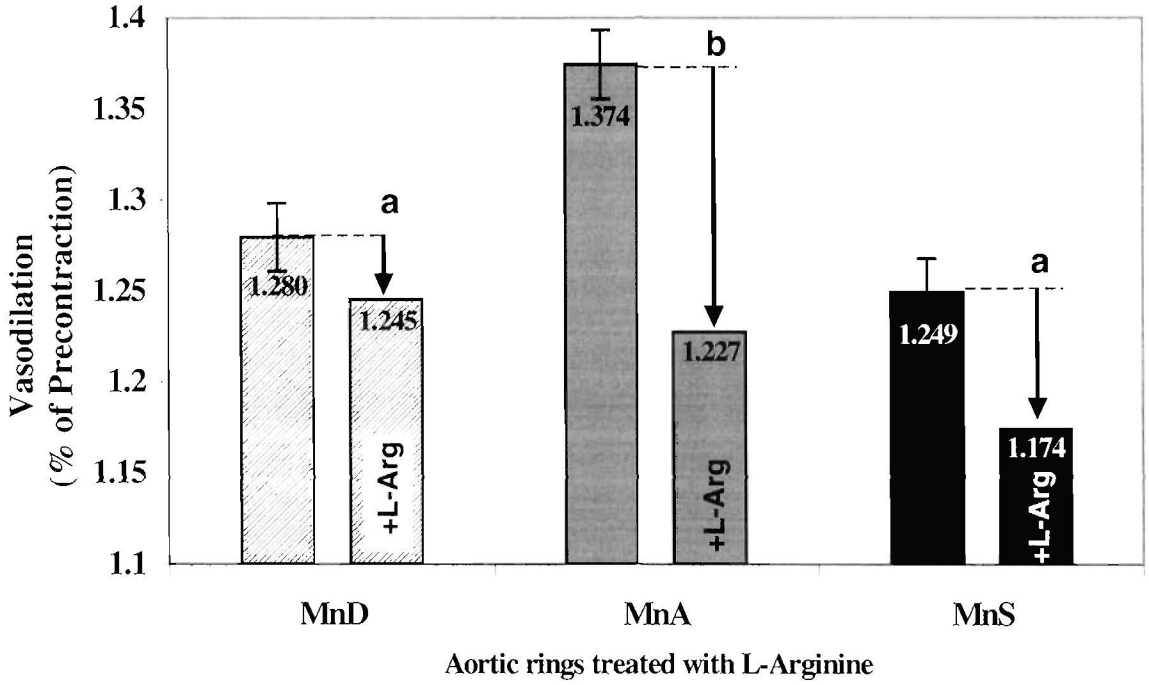
(a, b) Different letters on the top of the arrows depict statistically significant differences on the length of the arrows, which symbolizes the reduction in vessel sensitivity to Ach after the addition of the NOS inhibitor substrate (L-NMMA) in each individual diet group.

4.3.3. Effects of Dietary Mn on Vasodilation after in vitro Supplementation with L-Arginine

Pretreatment of the rings with the NOS substrate L-arginine did not reverse the previously observed decreases in Ach-induced vasodilation in the MnD and MnS groups when they were compared to the MnA group (Figures 4.5 and A.4). The differences in vasodilatory response (Figures 4.5) and vessel sensitivity to Ach (Figures 4.6) appear to be significant, when we compare the effect of the addition of L-Arginine in each diet group separately (comparison of arrows in the above figures).

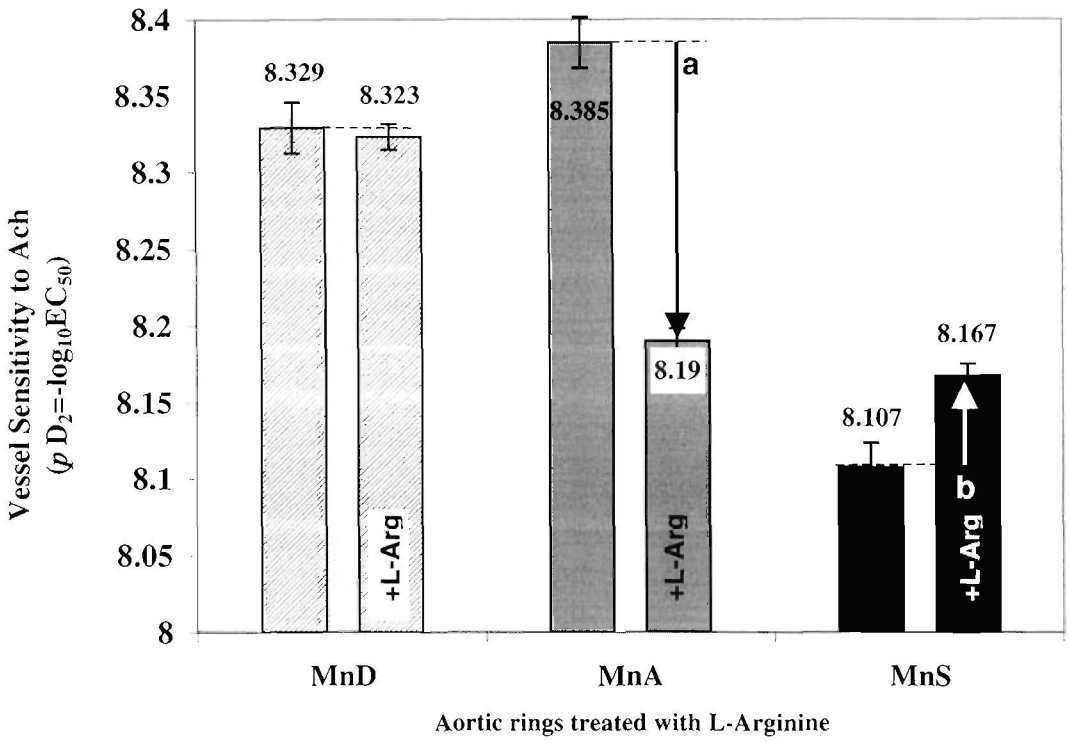
However, when L-arginine is added in the tissue bath during the preconditioning/ equilibration stage, the percent of reduction of Ach-induced vasodilation includes the effect of L-arginine on the basal NO release (since L-arginine is added before the application of the Ach-cumulative dose response curve). That means that any decreases in Ach-induced vasodilation are of limited biological significance and only an increase in vasodilation would provide useful information for the interpretation of the effect of Mn on this part of the pathway. Thus, our data indicate that dietary Mn intake in the concentrations studied does not seem to eliminate the availability L-arginine, the substrate for NO synthesis.

Figure 4.5. Effects of dietary Mn on the maximum Ach-induced vasodilation (expressed as % of precontraction) after *in vitro* supplementation with L-Arginine in Sprague Dawley rat aorta.



(a, b) Different letters on the top of the arrows depict statistically significant differences on the length of the arrows, which symbolizes the reduction in Ach-induced vasodilation after the addition of the substrate (L-Arginine) in each individual diet group.

Figure 4.6. Effects of dietary Mn on the vascular sensitivity (pD_2) to Ach after treatment with NO substrate (L-Arginine) in Sprague Dawley rat aorta.

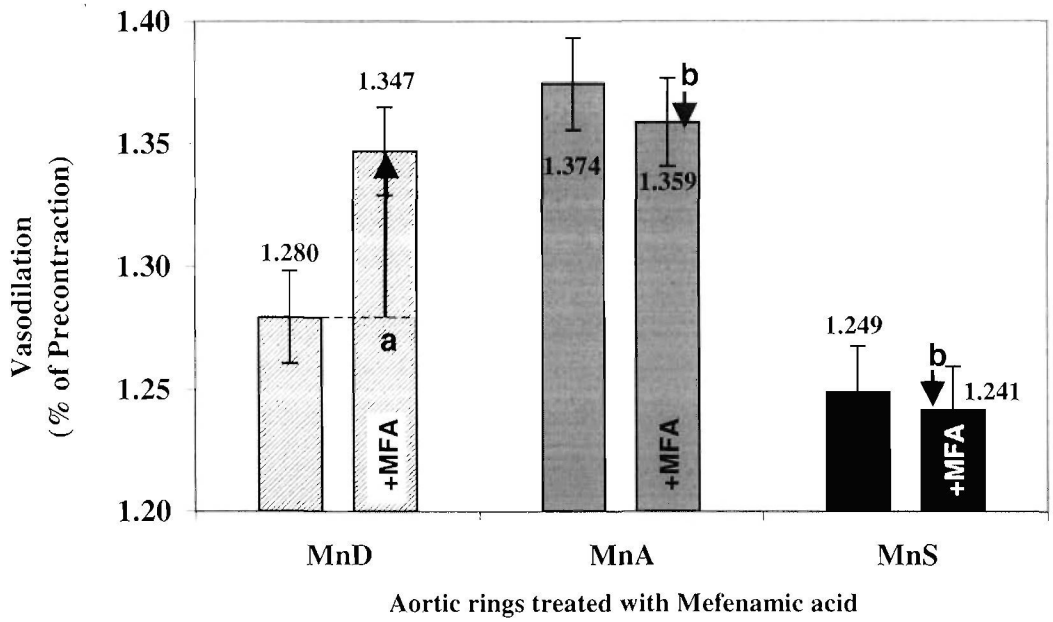


(a, b) Different letters on the top of the arrows depict statistically significant differences on the length of the arrows, which symbolizes the reduction in vessel sensitivity to Ach after the addition of the substrate (L-Arginine) in each individual diet group.

4.3.4. Effects of Dietary Mn on COX Pathway

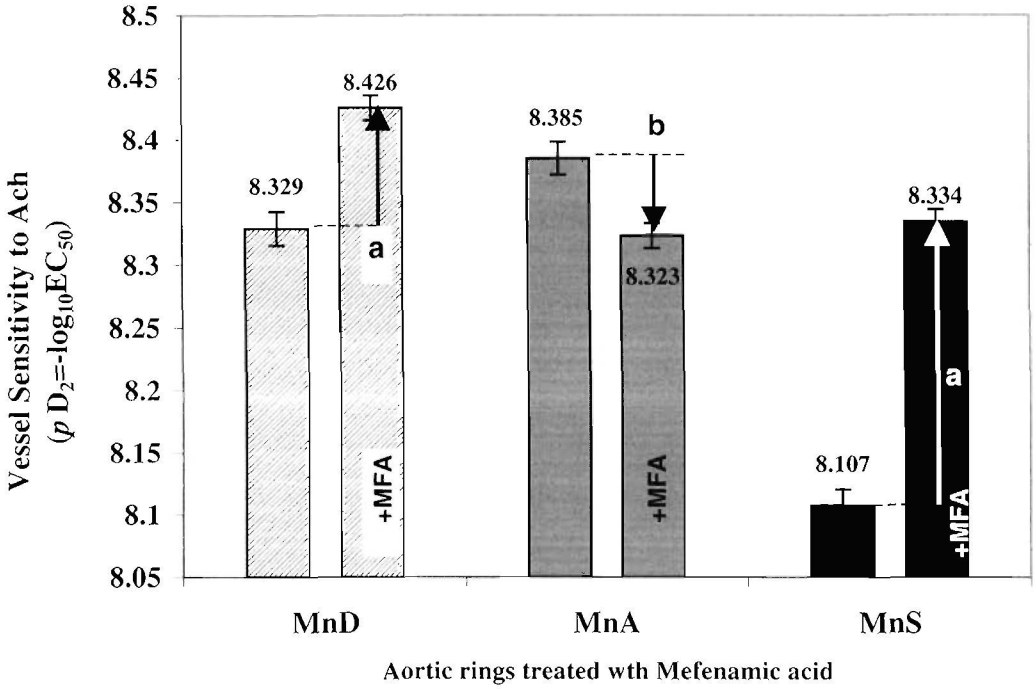
In MnD animals treated with the COX inhibitor MFA we observed an increased response to Ach-induced vasodilation, when compared to rings with no inhibitor from the same diet group (1.347 *vs.* 1.280) (Figure 4.7 and A.5). In the presence of MFA, vessel sensitivity of MnS rings to Ach was similar to that of the Control group (8.334 *vs.* 8.323), while MnD vessel sensitivity was significantly increased (8.426 *vs.* 8.323) (Figure 4.8). When compared to the rings in which there was no inhibitor added, the presence of MFA increased the vessel sensitivity to Ach in MnD and MnS rings but not in the MnA rings (depicted by arrows a and b in Figure 4.8). The enhancement of vasodilation after the addition of MFA (Figure 4.8) was significantly higher in MnD and MnS animals when compared to Controls (small decrease was observed). The addition of MFA increased vasodilation in MnD group thus, the presence of Mn deficiency seems to affect COX-mediated vasoactive component(s) which function(s) as endothelium-derived vasoconstrictor(s). The increased vessel sensitivity to Ach in MnS group does not appear to affect vasodilation (maximum % of vasodilation). Therefore, even though there is an indication for the production of an endothelium-derived vasoconstrictor in MnS animals, this effect might be masked or impaired probably due to the enhanced VSM pre-contraction in these rings (refer to chapter 2) and our observations are limited to provide an interpretation of the above findings.

Figure 4.7. Effects of dietary Mn on the maximum Ach-induced vasodilation (% Precontraction) after treatment with cyclooxygenase inhibitor (MFA) in Sprague Dawley rat.



(a, b) Different letters on the top of the arrows depict statistically significant differences on the length of the arrows, which symbolizes the reduction in Ach-induced vasodilation after the addition of the cyclooxygenase inhibitor (MFA) in each individual diet group.

Figure 4.8. Effects of dietary Mn on the vascular sensitivity (pD_2) to Ach after treatment with cyclooxygenase inhibitor (MFA) in Sprague Dawley rat.

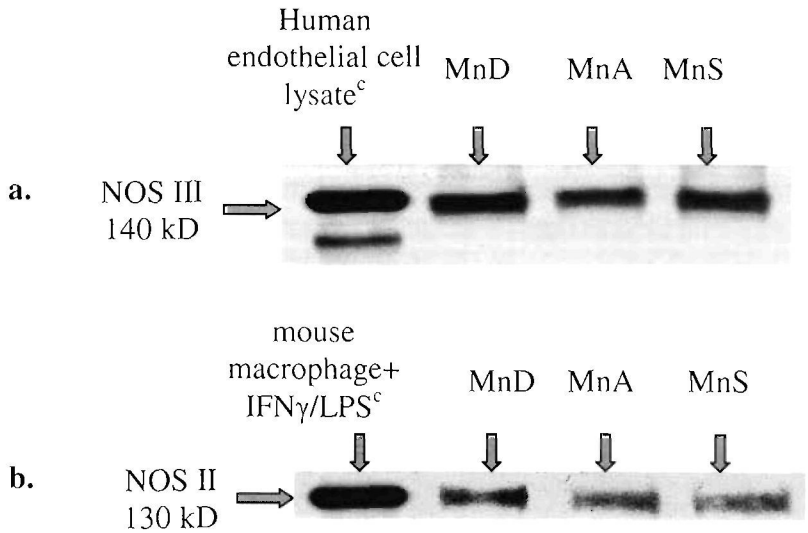


(a, b) Different letters on the top of the arrows depict statistically significant differences on the length of the arrows, which symbolizes the reduction in vessel sensitivity in Ach after the addition of the cyclooxygenase inhibitor (MFA) in each individual diet group.

4.3.5. Effects of Mn on NOS Expression

Determination of NOS expression allows us to detect whether the effect of dietary Mn on vasodilation is on the expression of one or both of NOS isoforms or if it acts at some point downstream to decrease NO production or bioactivity/ bioavailability. Quantification of the staining of each blot on the scanned film images was obtained by estimating the % of absorbance in each blot compared to the positive control blot (mouse macrophage+IFNγ/LPS for NOS II and human endothelial cell lysate for NOS III) that was used in each transfer. The increased variability among diet groups did not allow us to compare the statistical significance of the findings, however, we did not observe any differences in the NOS II nor NOS III expression among diet groups as appears in Figure 4.9a and 4.9b.

Figure 4.9. Two representative western blot determinations of the relative amounts of NOS III (a) and NOS II (b) among diet groups.



[c] Human endothelial cell lysate was used as a standard (positive control) for NOS III and mouse macrophage + IFNγ/ LPS as a standard for NOS II.

4.4. Discussion

Manganese deficiency was confirmed by the suppression of animal growth in the MnD diet group, as well as by the lower hepatic Mn content (Table 4.1). Retardation of rodent growth in Mn deficiency is an effect of reduced efficiency of food conversion with the dietary Mn depletion and has been reported in the past [Yang et al, 1998][Yang et al, 1998b] [Kalea et al, 2005][Taylor et al, 1997][Klimis-Tavantzis et al, 1993][Yang et al, 1998][Yang et al, 1998b][Paynter, 1980][Fahim et al, 1990][Malecki et al, 1994]. The decrease in liver weight in MnD rats was proportional to the decrease in body weight in MnD animals (Table 4.1). Hepatic Mn concentration in liver was significantly reduced in the MnD diet group when compared to MnA/ Control, while there were no differences between MnA and MnS animals (Table 4.1).

We document for the first time that the effect of dietary Mn on endothelium-dependent vasodilation of the rat aortic vessel is independent of the NOS expression and NO synthesis. The reduced vasodilation observed in MnD and MnS animals when compared to Controls is not an effect of substrate availability or expression of NOS. Altering the Mn from control levels in the experimental diets in both directions (Mn deficiency and Mn supplementation) modified vasodilation in response to Ach, which was affected by the presence of endothelium. In MnD diet we observed a potentiation of vasodilation to Ach with the presence of a COX inhibitor (MFA), which is an indication of synthesis of a COX-dependent endothelium-derived contracting factor in aortas of MnD animals. In MnS animals vessel sensitivity to Ach was increased when COX was inhibited, however that had no effect on the maximum vasodilation.

There have been several reports that COX inhibition increases the response to Ach in hypertensive patients while it has no effect on Ach-induced vasodilation in normal patients [Laemmel et al, 2003] and that COX derivatives can curtail endothelial responses in essential hypertension [Taddei et al, 1997]. In spontaneously hypertensive rats, an impairment of endothelium dependent relaxation was associated with an increased release of TXA_2, a vasoconstrictor eicosanoid underlying the endothelial cell dysfunction in hypertension [Kato et al, 1990].

It has been recently reported that dietary Mn does not affect the expression of NOS III in rat aorta when the latter was estimated by immunohistochemical analyses. [Ensunsa et al, 2004]. Comparisons were conducted only between MnD and MnS (MnS was the Control group in the study of Ensunsa et al, 2004). There have been no studies to date on the NOS II expression in different dietary intakes of Mn, nor on the activity of the two isoforms. We know that possible changes in activity of NOS II and III are independent of the expression of the two enzymes. NOS III activity is markedly inhibited by NO itself, while NOS II is resistant to NO inhibition. Thus, even if we were able to observe changes in enzyme expressions in our study, we could not assume that this has an effect on their activity in the same groups. At sites of endothelial damage, locally released cytokines stimulate iNOS expression and activity in the vascular wall and the NO produced reduces eNOS activity even though the expression of the enzyme might be similar or higher [Cernadas et al, 1998].

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 [Zidenberg-Cherr, 1983]. Deficiency of Mn is

known to reduce the activity of MnSOD in liver, heart and kidney, allowing the formation of superoxide [Ensunsa et al, 2004][Malecki et al, 1994][Malecki et al, 1996][Paynter DI, 1980]. This deleterious free radical reacts with and inactivates NO. An increase in formation of NO by-products such as peroxynitrite (ONOO-), nitrite (NO_2) and nitrate (NO_3) is an indication of NO inactivation. Superoxide dismutase has been shown to potentiate the effects of the release of basal [Rubanyi & Vanhoutte, 1986] and Ach-induced NO-dependent relaxation in arterial rings [Mian & Martin, 1995], with basal NO activity being more sensitive to inactivation by superoxide anion than Ach-stimulated activity [Mian & Martin, 1995]. 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 has been shown in the past for other trace mineral deficiencies such as selenium (Se) [Weaver et al, 2001]. Bovine aortic endothelial cells have been reported to produce higher TXA_2 (vasoconstrictor) and lower PGI_2 (vasodilator) in Se deficiency, which has been associated with aging but most importantly is a pathological indicator in children and adolescents with a predisposition in coronary heart disease [Mihai et al, 1992][Akimova et al, 1994].

Our data suggest that Mn deficiency appears to shift the balance of endothelium-derived eicosanoids by leading to a release of an endothelium-derived vasoconstrictor, possibly TXA_2. This finding suggests that Mn deficiency might have a profound effect in the elderly as well as in populations that are prone to cardiovascular disease. Even though there is limited information on the mechanisms underlying the modifications in eicosanoid pathway by the presence of dietary Mn, we do know that patients with congestive heart failure due to ischemic heart disease or cardiomyopathy have 82.8%

insufficient intake of dietary Mn [Gorelik et al, 2003] while patients with atherosclerosis appear to have decreased concentrations of Mn in aorta, liver, myocardium, adrenal glands, pancreas and kidney when compared to healthy individuals [Volvkov, 1962]. The presence of Mn might modulate the synthesis or activity of key enzymes in the complicated COX pathway. In Se deficiency the activity of PGI_2 synthase is downregulated by the increased oxidative stress, which might be the case in Mn deficiency as well [Weaver et al, 2001].

The product of the antioxidant effect of Mn+2 is Mn+3, which might form Mn+4 and Mn+2 in the presence of water and subsequently a two-electron oxidation by Mn+5 might take place [Coassin et al, 1992]. Formation of Mn in higher oxidation states accounts for the pro-oxidant role of Mn that might lead to an increased oxidative stress state, even though the 45-50 ppm are far from the toxic Mn levels (~1000ppm). A small increase in oxidative stress in MnS diets might explain the increased vessel sensitivity of MnS aortas to Ach in the presence of MFA. Such an increase might be an indication of the presence of an endothelium-mediated vasoconstrictor such as TXA_2, whose effect on inhibiting maximum vasodilation in possibly blunted by the increased VSM constriction observed in MnS rings (Chapter 2).

Dietary Mn seems to affect endothelium-mediated vasodilation both in deficiency and supplementation states. Even though this effect is independent of NO synthesis and generation, it is possible that it affects the bioavailability of NO in downstream endothelial pathways. The presence of oxidative stress in Mn deficiency due to the reduced activity of the major antioxidant enzyme MnSOD might reduce vasodilation by affecting the degradation of NO after it is produced or by enhancing the production of

endothelium-derived vasoconstrictors such as TXA_2, with the concomitant inhibition of endothelium-derived vasodilators such as PGI_2. The findings provide further information on the effects of Mn deficiency on oxidative stress and vascular dysfunction.

Chapter 5

Summary and Conclusions

Manganese is a multifunctional trace element involved in a plethora of metabolic pathways. It is a cofactor for enzymes such as MnSOD, arginase, glycosyltransferases and sulfonases and it has been reported to antagonize or substitute for several divalent ions in the cellular environment. Thus, Mn may be crucial in the modulation cellular functions that regulate vascular tone.

5.1. Manganese and Vasoconstriction

We document for the first time that the presence of Mn in the diet affects the α_1 adrenergic agonist-induced contractions of the rat aortic vessel. This trace element seems to affect both the maximal contractile response of the aorta to Phe and the membrane-related receptor sensitivity. Supplementation of Mn in 45-50 ppm, which is far from the toxic levels, did not affect the sensitivity of the adrenergic receptors in the endothelial and VSM cells, even though it significantly increased the VSM constriction. In Mn deficiency, we observed an increase in adrenergic receptor sensitivity (higher pD_2) when the endothelium was present, when compared to the control diet group, which did not affect the responsiveness of the contractile machinery (Fmax). The hypocontractility of intact rings from MnS group was attributed in part to the increased basal vasodilator tone of the endothelial cell layer, which balances the increased constriction of the vascular smooth muscle (as observed in disrupted rings). We concluded that Mn affects endothelial cells blunting the enhancement of Phe-induced vasoconstriction caused by the

presence of Mn in VSM. The enhanced effect or role of Mn on the contractile efficiency of the VSM is not on the receptor level but rather on the contractile machinery.

In our study we also examined vascular responsiveness to adrenergic stimulation of animals fed diets with different concentrations of Mn. We examined the α_1 adrenergic receptor mediated vasoconstriction using L-Phe, a synthetic catecholamine that causes VSM contraction by stimulating the release of calcium from intracellular stores. We hypothesized that the presence of Mn in the diet inhibits the vascular contractions induced by antagonizing Ca^{+2} in the agonist-induced vasoconstriction [Liu et al., 2002][Yan et al., 1998][Yan et al., 2001][Salaices et al., 1983][Kuribayashi, 1969][Sullivan et al., 1968]. Our findings are supported by similar *in vitro* observations on the effect of different concentrations of Mn on adrenergic receptor stimulated vasoconstriction in porcine coronary artery, goat and human cerebral arteries as well as rat aorta [Yan et al., 1998][Yan et al., 2001]. Manganese can pass through voltage-dependent Ca^{+2} channels and might share a Ca^{+2}-blocking property in smooth muscle cells by antagonizing Ca^{+2} and preventing its binding to calmodulin for the activation of NOS and the basal production of NO. Finally, the enhancement in maximum VSM constriction in the MnS diet group when compared to control indicates that the presence of Mn enhances the efficiency of the contractile machinery in the VSM and could imply an effect of the trace element on the cross bridge formation of the smooth muscle.

In our study we did not observe any differences in vasoconstriction of MnD rings when compared to control rings (MnA), even though there was an increased vascular sensitivity to the adrenergic agonist (higher pD_2 of intact MnD rings but not of disrupted). However, we did observe a decreased constriction of the VSM in

endothelium-disrupted MnD rings only, when compared to the control group, which was independent of the vascular smooth muscle cell sensitivity. This may suggest an impaired ability of the endothelial cell in MnD animals to synthesize vasodilators or protect their integrity in order to counterbalance vasoconstriction, which may be explained by the increased oxidative stress and decreased NO bioavailability in case of Mn deficiency.

Our results demonstrate, for the first time, that dietary Mn is implicated in the vascular contractile model, affecting the mechanical properties of blood vessels in response to an adrenergic stimulus, which implies alterations in regulation of vascular tone. The effects of Mn depend on its concentration in the diet and on the presence of a viable endothelium.

5.2. Manganese and Vasodilation

We documented that the presence of Mn in the diet affects the function of the endothelial layer since we did observe significant differences among diet groups primarily in endothelium-dependent vasodilation (induced by Ach). The alterations observed in endothelium-independent vasodilation as induced by a direct smooth muscle relaxant (SNP) were of small biological significance (Chapter 3). Mn deficiency seems to reduce the production and/or bioactivity of endothelium-generated vasodilators, without affecting membrane-related events. Dietary Mn has no effect on enzyme (NOS) expression nor substrate (L-arginine) availability that would regulate NO formation. Our findings are in accordance with previous determinations of NOS III expression in different dietary intakes of Mn. However, we can not exclude the possibility that dietary Mn might affect the bioavailability of NO after it is produced (basal or receptor-

stimulated NO release). Manganese is known to affect the synthesis of metalloenzymes such as MnSOD that protects the cell membrane structure from free radicals (O^{2-} generation) generated by cellular respiration [Malecki et al., 1994] [Ensunsa et al., 2004] [Zanetti et al., 2001][Wambi-Kiesse et al., 1999]. It is now known that oxidative stress initiates endothelial dysfunction by affecting the production of endothelium-derived vasoactive mediators [Gao et al., 1994][Williams et al., 1998][Matsuoka, 2001]. Decreased bioavailability of endothelial-released NO due to its increased degradation by superoxide affects the biomechanical properties of the contractile machinery.

Manganese is also a metalloenzyme for arginase, the enzyme that catalyzes the formation of L-ornithine for L-arginine, competing for the same substrate (L-arginine) with NOS III which catalyzes the formation of L-citrulline from L-arginine with the subsequent production of NO. Mn deficiency has been reported to affect arginase activity and also NO production and/or bioavailability. A competition between arginase and endothelial NOS has been suggested as a novel mechanism for atherosclerotic endothelial dysfunction [Ming et al., 2004]. There are no studies to our knowledge on the arginase activity in vascular wall in Mn deficient rats. A reduced activity of arginase in aortic endothelial cells of MnD animals would enhance NOS activity in order to form more NO from L-arginine. However, our data indicate that dietary Mn does not seem to affect the synthesis of NO from L-arginine, which could be attributed to a less severe induction of Mn deficiency in our experiments (compared to Mn deficiency induced in offsprings of MnD rats).

When Mn is present in levels of supplementation (MnS) we observed a decrease in vessel sensitivity to Ach. Such a finding suggests an effect of dietary Mn on the

relationship between Ach and the membrane receptor of the agonist or a decrease in the release of NO from endothelial cells due to increased degradation of NO attributed to the pro-oxidant nature of the trace mineral. It has been well documented in the past that dietary Mn affects the concentration and synthesis of proteoglycan and glycosaminoglycan (GAG) structures in rat aorta, which interact in divergent ways with biologically effective molecules, such as receptors, enzymes, cytokines, growth factors and proteins [Yang et al., 1998][Yang et al., 1998b][Klimis-Tavantzis et al., 1989] [Wight et al., 1992]. Sulfonation is the recognized primary mechanism that influences biological activity by determining high specificity in these receptor-ligand binding interactions [Strott, 2002][Lyon et al., 1998]. Overexpression of HSPGs appears to enhance vascular sensitivity to agonists, an indicator of the binding affinity of the ligand with the specific receptor, especially in low ligand concentrations [Risler et al., 2002][Kramer et al., 2003]. We observed a decrease in the sulfation of HS disaccharides in MnS aortas [Kalea et al, 2004]. This has been related to a downregulation of gene expression and clustering (aggregation) of Ach receptors *in vitro* [Meier et al., 1998][Jones et al., 1996][McDonnel et al., 2004] and could explain the decreased vessel sensitivity to Ach in MnS animals, provided that we do have maximum receptor occupancy in our experimental conditions.

Ensunsa et al. [Ensunsa et al., 2004] recently reported a decreased vascular reactivity to Ach in aortic rings from rats fed a MnS diet, while they did not observe any statistically significant differences to SNP-induced vasodilation. In their experimental protocol they used MnD female offspring of MnD Sprague Dawley rats and compared them with MnS-fed rats (45 ppm) without using a MnA/ Control diet group. Our results

are in accordance with the findings of this study (reduced Fmax and pD_2 in MnS group when compared to MnD) [Ensunsa et al., 2004].

Altering the Mn from control levels in the experimental diets in both directions (Mn deficiency and Mn supplementation) modified vasodilation in response to Ach. This was affected by the presence of endothelium. In Mn deficiency the presence of a COX inhibitor potentiated vasodilation in response to Ach, which is an indication of synthesis of a COX-dependent endothelium-derived contracting factor in aortas of MnD animals possibly of TXA_2. Oxidative stress in the presence of a reduced MnSOD activity has been shown to affect the production of eicosanoids through the COX pathway. This has been documented in the past for other trace mineral deficiencies such as selenium (Se) [Weaver et al., 2001]. Increased production of endothelium-derived vasoconstrictors such as TXA_2 and decreased formation of endothelium-derived vasodilators such as lower PGI_2 (vasodilator) is common in elderly and is a pathological indicator in children and adolescents with a predisposition in coronary heart disease [Mihai et al., 1992][Akimova et al., 1994]. Thus, correction of a Mn deficiency in the elderly as well as in populations that are prone to cardiovascular disease is of extreme importance. The mechanisms underlying the modifications in eicosanoid pathway by the presence of dietary Mn are unknown and might depend on the role of Mn as a cofactor for enzyme systems that may participate in the complicated COX pathway. The role of Mn as a pro-oxidant may explain the possible release of an endothelium-mediated vasoconstrictor in MnS rings, whose effect seems to induce borderline changes in vasodilation or may be masked by the increased VSM constriction observed in MnS rings.

Dietary Mn seems to affect endothelium-mediated vasodilation both in deficiency and supplemented states. Even though this effect is independent of NO synthesis and generation, it is possible that it affects the bioavailability of NO in downstream endothelial pathways. The presence of oxidative stress in Mn deficiency due to the reduced activity of the major antioxidant enzyme MnSOD might decrease vasodilation by affecting the degradation of NO after it is produced. Additionally, it may enhance the production of endothelium-derived vasoconstrictors such as TXA_2., with the concomitant inhibition of endothelium-derived vasodilators such as PGI_2. These findings provide further information on the effects of Mn deficiency on oxidative stress and vascular dysfunction and are in agreement with all previous investigator's results, complementing the available information on the effect of different dietary Mn intakes.

5.3. Limitations and Recommendations for Future Work

Our studies concluded that Mn has an effect on endothelium-mediated vasodilation, which depends on the Mn intake in the diet. The presence of the trace element dose not affect the synthesis and generation of NO in the vascular cell nor the cGMP pathway in the VSM cells, since all diet groups had the same response to NO donors (SNP). Our data could not exclude the possibility of a reduced vasodilatory role of the endothelium at least in Mn deficiency. A reduced NO bioavailability may be a result of decreased formation or increased degradation of the diffusible vasodilator. In our study we described the effect of Mn intakes on NO generation. Further studies on the concentration of NO by-products such as ONOO-, NO_2 and NO_3 are necessary in order

to detect possible increase in NO degradation due to oxidative stress, which seems to describe a Mn deficiency condition.

Manganese seems to affect the gene expression or activation (clustering or phosphorylation) of the receptors that were used in our experiments. This seems to be mediated by the effect of Mn on glycosaminoglycan concentration and structure, which participate in receptor structures in order to facilitate the ligand-receptor binding. It would be interesting to study in the future whether the presence of Mn in the diet affects the receptor molecules and the glycosaminoglycan-receptor binding capacities.

Additionally, since dietary Mn intake may modulate EC gene expression, it would be interesting to study the effect of the different diets on the expression of one or more ''atheroprotective genes'' in EC, such as MnSOD, COX-II and NOS III, whose antioxidant, antithrombotic, and antiadhesive properties are consistent with a protective role in the atherosclerotic disease.

The deficiency induced in our study by elimination of dietary Mn from food and water can not be considered a severe deficiency. It is interesting to note that Mn deficiency at <1 ppm in weanling animals for a short time period affects vascular responses to agonists. Although deficient Mn status in humans is rare, subclinical Mn deficiency in humans has been noted [Pennington et al., 1991] and it usually underlines several pathologic conditions [Volvkov, 1962][Gorelik et al, 2003]. Our findings indicate that in Mn deficiency we have a production of endothelium-derived vasoconstrictor prostanoids, and a decrease in endothelium-derived vasodilators suggesting that an underlying deficiency of the trace mineral may exacerbate the clinical profile of the above diseases.

Manganese is a multifunctional trace element participating in many fundamental processes in the cell [Korc M, 1993]. Its effect on the vascular system, such as the control of vasomotor tone and blood pressure regulation still remains to be unraveled. Our results demonstrate, for the first time, that dietary Mn affects different pathways that regulate the functional properties of the rat aorta, which might be significant for populations with predisposition to cardiovascular diseases. This effect depends on its concentration in the diet and on the presence of a viable endothelium. The adequate dietary intake of Mn as suggested by AIN (10-15 ppm) seems to be the optimum dietary intake for growth as well as for normal vascular function. However, the effect of different dietary Mn concentrations between 10 ppm and 45 ppm (which has been used in literature as a control group) on vascular function and arterial functional properties need to be evaluated.

BIBLIOGRAPHY

Abeywardena, M.Y., Jablonskis, L.T., & Head, R.J. (2002). Age- and Hypertension-induced changes in abnormal contractions in rat aorta. J Cardiovasc Pharmacol. 40:930-7.

Adeagbo, A.S.O., & Triggle, C.R. (1993). Interactions of nitric oxide synthase inhibitors and dexamethasone with a-adrenoreceptor-mediated responses in rat aorta. Br J Pharmacol. 109:495-501.

Akimova, E. V. (1994). Prostacyclin and thromboxane A2 levels in children and adolescents with an inherited predisposition to coronary heart disease: a family study. Coron Artery Dis. 5:761-765.

Allan, G., Brooks, C.D., Cambridge, D., & Hladkiwsky, J. (1983). Enhanced responsiveness of vascular smooth muscle to vasoconstrictor agents after removal of endothelial cells. Br J Pharmacol. 79:334P.

American Heart Association. (2003). Heart Disease and Stroke Statistics – 2004 Update, Dallas, Tex.: American Heart Association.

Angus, J.A., & Wright, C.E. (2000). Techniques to study the pharmacodynamics of isolated large and small blood vessels. J Pharmacol Toxicol Methods. 44:395-407.

Arnal, J.F., Dinh-Xuan, A.T., Pueyo, M., Darblade, B., & Rami, J. (1999). Endothelium-derived nitric oxide and vascular physiology and pathology. Cell Mol Life Sci. 55: 1078-87.

Asbun-Bojalil, J., Castillo, E.F., Escalante, B.A., & Castillo, C. (2002). Does segmental difference in a1 adrenoreceptor subtype explain contractile difference in rat abdominal and thoracic aortae? Vascul Pharmacol. 38:169-75.

Barceloux, D.G. (1999). Manganese. Clinical Toxicology. 37:293-307.

Beach, P.K., Spain, D.A., Kawabe, T., Harris, P.D., & Garrison, R.N. (2001). Sepsis increases NOS-2 activity and decreases Non-NOS mediated acetylcholine-induced dilation in rat aorta. J Surg Res. 96:17-22.

Bloodsworth, A., O'Donnell, V., & Freeman, B.A. (2000). Nitric oxide regulation of free radical and enzyme mediated lipid and lipoprotein oxidation. Arterioscler Thromb Vasc Biol. 20:1707-15.

Boulanger, C.M.J. (1999). Endothelial Dysfunction: a novel therapeutic target: Secondary endothelial dysfunction: Hypertension and Heart Failure. J Mol Cell Cardiol. 31:39-49.

Cai, H., & Harrison, D.G. (2000). Endothelial dysfunction in cardiovascular diseases: The role of oxidant stress. Circ Res. 87:840-4.

Carrier, G.O., & White, R.E. (1985). Enhancement of alpha-1 and alpha-2 adrenergic agonist-induced vasoconstriction by removal of endothelium in rat aorta. J Pharmacol Exp. Ther. 232:682-7.

Cernadas, M.R., Sanchez de Miguel, L., Garcia-Duran, M., Gonzalez-Fernandez, F., Millas, I., Monton, M., Rodrigo, J., Rico, L., Fernandez, P., de Frutos, T., Rodriguez-Feo, J.A., Guerra, J., Caramelo, C., Casado, S., & Lopez-Farre. (1998). Expression of

constitutive and inducible nitric oxide synthases in the vascular wall of young and aging rats. Circ Res. 83:279-86.

Chamiot-Clerc, P., Colle, M.H., Legrand, M., Sassard, J., Safar, M.E., & Renaud, J.F. (1999). Evidence for a common defect associated with pressure in the aorta of two hypertensive rat strains. Clin Exp Pharmacol Physiol. 26:883-8.

Clarkson, T.W. (1993). Molecular and ionic mimicry of toxic metals. Annu Rev Pharmacol Toxicol. 32:545-71.

Collins, G.A., Sutter, M.C., & Teiser, J.C. (1972). The effects of manganese on the rabbit anterior mesenteric portal vein. Can J Physiol Pharmacol. 50:300-9.

Cotzias, G.C. (1958). Manganese in health and disease. Physiol Rev. 38:503.

Cox, D.A., & Cohen, M.L. (1996). Effects of oxidized low-density lipoprotein on vascular contraction and relaxation: clinical and pharmacological implications in atherosclerosis. Pharmacol Rev. 48:3-19.

Deth, R., & Lynch, C. (1981). Inhibition of α-receptor-induced Ca^{+2} release and Ca^{+2} influx by Mn^{+2} and La^{+2}. Eur J Pharmacol. 71:1-11.

De Vriese, A.S., Verbeuren, T.J., Van de Voorde, J., Lameire, N.H., & Vanhoutte, P.M. (2000). Endothelial dysfunction in diabetes. Br J Pharmacol. 130:963-74.

Doisey, E.A. (1972). Micronutrient controls of biosynthesis of clotting proteins and cholesterol. In Trace Substances in Environmental Health (Hemphill, D., ed.), vol. 6, pp. 193-99. University of Missouri, Columbia.

Dorman, D.C., Struve, M.F., James, R.A., McManus, B.E., Marshall, M.W., & Wong, B.A. (2001). Influence of dietary manganese on the pharmacokinetics of inhaled manganese sulfate in male CD rats. Toxicological Sciences. 60: 242-51.

Drexler, H., & Hornig, B. (1999). Endothelial dysfunction in human disease. J Mol Cell Cardiol. 31:51-60.

Dupont, C.L., & Tanaka, Y. (1985). Blood manganese levels in children with convulsive disorder. Biochem Med. 33:246-55.

Edwards, J.G., Hameed, H. & Campbell, G. (1988). Induction of fibroplast spreading by Mn+2: apossible role for unusual binding sites for divalent cations in receptors for proteins containing Arg-Gly-Asp. J Cell Science. 89:507-13.

Ekanayake, R.P., & Klimis-Tavantzis, D.J. (1995). The effect of dietary manganese on the ultrastructure of aorta and liver tissues. FASEB J. 9:A447.

Ensunsa, J.L., Schrader, H.R., & Keen, C.L. (2002). The effect of a reduction in arginase activity on production of nitric oxide metabolites in manganese deficient rats. FASEB J. 16:A272.

Ensunsa, J.L., Symons, J.D., Lanque, L., Schrader, H.R., & Keen, C.L. (2004). Reducing arginase activity *via* dietary manganese enhances endothelium-dependent vasorelaxation of rat aorta. Exp Biol Med. 229:1143-53.

Fahim, F.A., Morcos, N.Y.S., & Esmat, A.Y. (1990). Effects of dietary magnesium and/or manganese variables on the growth rate and metabolism of mice. Ann Nutr Metab. 34:183-92.

Félétou, M. & Vanhoutte, P.M. (2000). Endothelium-dependent hyperpolarization of vascular smooth muscle. Acta Pharmacol Sin. 21:1-18.

Fernandes, A.C. & Manso, C.F. (1988). Copper-manganese interactions concerning red cell and plasma lipid peroxidation. Free Rad Res Comms. 5:177-84.

Fleisch, J.H. (1974). Pharmacology of the aorta: A brief review. Blood Vessels. 11:193-211.

Freeland-Graves, J.H. (1988). Manganese: An essential nutrient for humans. Nutrition Today. Nov-Dec:13-9.

Freeland-Graves, J.H., & Llane, C. (1994). Models to study manganese deficiency. In: Manganese in Health and Disease (Klimis-Tavantzis D.J., ed.) pp.2-37. CRC Press, Inc. Boca Raton, FL.

Friedman, B.J., Freeland-Graves, J.H., Bales, C.W., Behmardi, F.B., Shorey-Kutschke, R., Willi, R.A., Crosby, J.B., Trickett, P.C. & Houston, S.D. (1988). Manganese balance

and clinical observations in young men fed a manganese deficient diet. J Nutr. 117:133-43.

Furchgott, R.F., & Zawadzski, J.V. (1980). The obligatory role of the endothelial cells in relaxation of arterial smooth muscle by acetylcholine. Nature. 288:373-6.

Gao, H., Korthuis, R.J., & Benoit, J.N. (1994). Effects of reactive oxygen metabolites on norepinephrine-induced vasoconstriction, Free Rad Biol Med. 16:839-43.

García-Sáinz, J.A., Vázquez-Prado, J., & Medina, L.C. (2000). Alpha 1-adrenoceptors: function and phosphorylation. Eur. J. Pharmacol. 389:1-12.

Gorelik, O., Almoznino-sarafian, D., Feder, I., Wachsman, O., Alon, I., Litvinjuk, V., Roshovsky, M., Modai, D., & Cohen, N. (2003). Dietary intake of various nutrients in older patients with congestive heart failure. Cardiology. 99:177-81.

Govers, R., & Rabelink, T.J. (2001). Cellular regulation of endothelial nitric oxide synthase, Am J Physiol Renal Physiol. 280:F193-206.

Gropper, S.S., Smith, J.L., & Groff, J.L. (2005). Advanced Nutrition and Human Metabolism, 4th Edition, Thomson Wadsworth, Belmont, CA, USA.

Harris, L.M., Faggioli, G.L., Shah, R., Koerner, N., Lillis, L., Dandona, P., Izzo, J.L., Snyder, B., & Ricotta, J.J. (1995). Vascular reactivity in patients with peripheral vascular disease. Am J Cardiol. 76:207-12.

Hoffman, B.B., Yim, S., Tsai, B.S., & Lefkowitz, R.J. (1981). Preferential uncoupling by manganese of alpha adrenergic receptor mediated inhibition of adenylate cyclase in human platelets. Biochem Biophys Res Commun. 100: 724-31.

John, S., & Schmieder, RE. (2000). Impaired endothelial function in arterial hypertension and hypercholesterolemia: potential mechanisms and differences. J Hypertens. 18:363-74.

Johnston, P.E., & Korynta, E.D. (1990). The effect of dietary protein source on manganese bioavailability to the rat. P.S.E.B.M. 195:230-6.

Jones, G., Herczeg, A., Ruegg, M.A., Lichtsteiner, M., Kröger, S., & Brenner, H.R. (1996). Substrate-bound agrin induces expression of acetylcholine receptor -subunit gene in cultured mammalian muscle cells. 93:5985-90.

Kalea, A.Z., Harris, P.D., & Klimis-Zacas, D. (2005). Dietary manganese suppresses α_1 adrenergic receptor mediated vascular contraction. J Nutr Biochem. 16:44-9.

Kalea, A.Z., Norton, C., & Klimis-Zacas, D. (2002). Dietary manganese affects the receptor signaling pathways of the vascular contractile machinery in rat aorta. J Trace Elem Experim Med. 14:16.

Kalea, A.Z., Norton, C., Harris, P.D., & Klimis-Zacas, D. (2003). Manganese (Mn) enhances endothelial modulation of α_1 adrenoreceptor (AR) mediated contraction of vascular smooth muscle (VSM) in rat aorta. F.A.S.E.B. J. 17:A335.

Kalea, A.Z., Lamari, F., Karamanos, N.K., & Klimis-Zacas, D. (2004). Dietary Manganese affects heparan sulfate concentration and sulfation pattern in Sprague-Dawley rat aorta, Federation of the European Connective Tissue Societies (FECTs) XIX, PB(26): 79.

Kappus, H., & Schenkman, J.B. (1979). Stimulatory and inhibitory effects of manganous and ferrous ions on epinephrine oxidation. Biochem Pharmacol. 28:1129-34.

Kasten, T.P., Settle, S.L., Misko, T.P., Currie, M.G., & Nickols, G.A. (1994). Manganese potentiation of nitric-oxide mediated vascular relaxation. Eur J Pharmacol. 253:35-43.

Kawabe, T., Harris, P.D., Zakaria, E.L., & Garrison, R.N. (2003). Sepsis alters vessel contraction by adrenoreceptor-induced nitric oxide and prostanoid. J Surg Res. 110:352-9.

Keen, C.L., Lonnerdal, B. & Hurley, L.S. (1984). Manganese. In: Biochemistry of the essential ultratrace elements (Frieden E, ed.) pp. 89-132. Plenum Press, New York, NY.

Klimis-Tavantzis, D., Taylor, P.N., Lewis, R.A., Flores, A.L. & Patterson, H.H. (1993). Effects of dietary manganese deficiency on high-density lipoprotein composition and metabolism in Sprague-Dawley rats. Nutr Res. 13:953-68.

Korc, M. (1993). Manganese as a modulator of signal transduction pathways. In: Essential and toxic trace elements in human health and disease: An Update. Willey-Liss, Inc. pp.235-55.

Kramer, K.L., & Yost, H.J. (2003). Heparan sulfate core proteins in cell-cell signaling, Annu Rev Genet. 37:461-84.

Krauss, G. (2001). Biochemistry of signal transduction and regulation Wiley-VCH, Weinheim, Germany.

Kuratko, C.N. (1997). Increasing dietary lipid and iron content decreases manganese superoxide dismutase activity in colonic mucosa. Nutrition and Cancer. 28:36-40.

Kuribayashi, R. (1969). Effects of Mn and Zn Ions on the contraction of smooth muscle of guinea pig Taenia Coli. Tohoku J Exp Med. 98:241-7.

Kwan, H.Y., Leung, P.C., Huang, Y., & Yao, X. (2003). Depletion of intracellular Ca+2 stores sensitizes the flow-induced Ca^{+2} influx in rat endothelial cells. Circ Res. 92:286-92.

Laemmel, E., Bonnardel-Phu, E., Hou, X., Seror, J., & Vicaut, E. (2003). Interaction between nitric oxide and prostanoids in arterioles of rat cremaster muscle in vivo. Am J Physiol Heart Circ Physiol. 285: H1254-60.

Leach, R.M. (1971). Role of manganese in mucopolysaccharide metabolism. Federation Proceedings. 30:991-4.

Liu, D.L., Yan, M., Chua, Y.L., Chen, C., & Lim, Y.L. (2002). Effects of molybdenum, silicon and nickel on alpha-1 adrenoreceptor induced constriction of rat isolated aorta. Clin Exp Pharmacol Physiol. 29:395-8.

Loscalzo, J., & Vita, J. (2000). Nitric oxide and the Cardiovascular System. Totawa, NJ: Humana Press.

Lucas, K.A., Pitari, G.M., Kazerounian S., Ruiz-Stewart, I., Park, J., Schulz, S., Chepenik, K.P., & Waldman, S.A. (2000). Guanylyl Cyclases and Signaling by Cyclic GMP. Pharmacol. Rev. 52:375-414.

Lüscher, T.F., & Vanhoutte, P.M. (1990). The endothelium modulator of cardiovascular function. Boca Raton, FL CRC Press. 1-215.

Lüscher, T.F. (1994). The endothelium and cardiovascular disease-a complex relation. N Engl J Med. 330:1081-3.

Lyon, M., & Gallagher, J.T. (1998). Bio-specific sequences and domains in heparan sulfate and the regulation of cell growth and adhesion. Matrix Biology. 17:485-93.

McIntyre, M., & Dominiczak, A.F. (1997). Nitric oxide and cardiovascular disease. Postgrad Med J. 73:630-4.

McNatt, M.L., Fiser, F.M., Elders, M.J., Kilgore, B.S., Smith, W.G., & Hughes, E.R. (1976). Uridine Diphosphate Xylosyltransferase activity in cartilage from manganese-deficient chicks. Biochem J. 160:211-6.

Malecki, E.A., Huttner, D.L., & Greger, J.L. (1994). Manganese status, gut endogenous losses of manganese and antioxidant enzyme activity in rats fed varying levels of manganese and fat. Biol Trace Elem Res. 42:17-29.

Martin, W., Furchgott, R.F., Villani, G.M., & Jothianandan, D. (1986). Depression of contractile responses in rat aorta by spontaneously released endothelium-derived relaxing factor. J Pharmacol Exp Ther. 237:529-38.

Matsuda, A., Kimura, M., Kataoka, M., Ohkuma, S., Sato, M., & Itokawa, Y. (1989). Quantifying manganese in lymphocytes to assess manganese nutritional status. Clin Chem. 35:1939-41.

Matsuoka, H. (2001). Endothelial dysfunction associated with oxidative stress in human. Diabetes Res Clin Pract. 54:S65-72.

Mayer, B., Pfeiffer, S., Schrammel, A., Koesling, D., Schmidt, K., & Brunner, F. (1998). A new pathway of nitric oxide/ cyclic GMP signaling involving S-Nitrosoglutathione. J Biol Chem. 273: 3264-70.

Mian, K.B., & Martin, W. (1995). Differential sensitivity of basal and acetylcholine-stimulated activity of nitric-oxide to destruction by superoxide anion in rat aorta. Br J Pharmacol. 115: 993-1000.

Michel, J.B., Feron, O., Sase, K., Praghakar, P., & Michel, T. (1997). Caveolin versus calmodulin. Counterbalancing allosteric modulators of endothelial nitric oxide synthase. J Biol Chem. 272:25907-12.

Mihai, K., Peto, J., Kolthay, J., Makary, A., & Timar, S. (1992). Balance of prostacyclin and thromboxane in offspring of parents with premature coronary arterial disease. Int J Cardiol. 35:13-8.

Militsopoulou, M., Lamari, F., & Karamanos, N.K. (2003). Capillary electrophoresis: a tool for studying interactions of glycans/proteoglycans with growth factors. J Pharm Biomed Anal. 32:823-8.

Molina, R., Hidalgo, A., & Garcia de Boto, M.J. (1992). Influence of mechanical endothelium removal techniques and conservation conditions on rat aorta responses. Methods Find Exp Clin Pharmacol. 14:91-6.

Mombouli, J.V., & Vanhoutte, P.M. (1999). Endothelial Dysfunction: A Novel Therapeutic target: Endothelial Dysfunction: From Physiology to Therapy. J Mol Cell Cardiol. 31:61-74.

Mombouli, J.V., Nakashima, M., Hamra, M., & Vanhoutte, P.M. (1999). Endothelium-dependent relaxation and hyperpolarization evokes by bradykinin in canine coronary arteries. Br J Pharmacol. 117:413-8.

Moncada, S., Palmer, R.M.J., & Higgs, E.A. (1989). Biosynthesis of nitric oxide from L-arginine: A pathway for the regulation of cell function and communication. Biochem Pharmacol. 38:1709-15.

Moncada, S., Palmer, R.M.J., & Higgs, E.A. (1999). Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacol Rev. 43:109-42.

Moncada, S., & Higgs, E.A. (1993). The L-Arginine - Nitric Oxide Pathway. New Engl J Med. 329:2002-12.

Mori, M., & Gotoh, T. (2000). Regulation of nitric oxide production by arginine metabolic enzymes. Biochem Biophys Res Commun. 275:715-9.

Nighorn, A., Byrnes, K.A., & Morton, D.B. (1999). Identification and characterization of a novel beta subunit of soluble guanylyl cyclase that is active in the absence of a second subunit and is relatively insensitive to nitric oxide. J Biol Chem. 274: 2525-31.

Nilius, B., & Droogmans, G. (2001). Ion channels and their functional role in vascular endothelium. Physiol Rev. 81:1416-59.

Pacaud, P., Loirand, G., Gregoire, G., Mironneau, C., & Mironneau, J. (1993). Noradrenaline-activated heparin-sensitive Ca^{2+} entry after depletion of intracellular Ca^{2+} store in portal vein smooth muscle cells. J Biol Chem. 268:3866-72.

Panza, J.A., Garcia, C.E., Kilcoyne, C.M., Quyyumi, A., & Cannon, R.O. III. (1995). Impaired endothelium-dependent vasodilation in patients with essential hypertension evidence that nitric oxide abnormality is not localized to a single signal transduction pathway. Circulation. 91:1732-8.

Papavasiliou, P.S., Miller, S.T., & Cotzias, G.C. (1966). Role of liver in regulating distribution and excretion of manganese. Am J Physiol. 211:211-6.

Patterson, K.Y., Holbrook, J.T., Bodner, J.E., Kelsay, J.L., Smith, J.C., & Veillon, C. (1984). Zinc, copper and manganese intake and balance for adults consuming self selected diets. Am J Clin Nutr. 40:1303-11.

Paynter, D. (1980). Changes in activity of manganese superoxide dismutase enzyme in tissues of the rat with changes in dietary manganese. J Nutr. 110:437-47.

Pennington, J.A., Young, B.E., Johnson, R.D., & Vanderveen, J.E. (1986). Mineral content of foods and total diets: the selected minerals in food survey, 1982 to 1984. J Am Diet Assoc. 86:876.

Pratt, W.B., & Taylor, P. (1990). Principles of drug action: the basis of Pharmacology. New York: Churchill Livingstone.

Price, S.A., Spain, D., Wilson, M.A., Harris, P.D., & Garrison, R.N. (1999). Subacute sepsis impairs vascular smooth muscle contractile machinery and alters vasoconstriction and dilator mechanisms. J Surg Res. 83:75-80.

Radomski, M.W., Palmer, R.M.J., & Moncada, S. (1990). Glucocorticoids inhibit the expression of an inducible but not the constitutive, nitric oxide synthase in vascular endothelial cells. P.N.A.S. U.S.A. 87:10043-7.

Rees, D.D., Cellek, S., Palmer, R.M.J., & Moncada, S. (1990). Dexamethazone prevents the induction by endotoxin of a nitric oxide synthase and the associated effects of vascular tone: an insight into endotoxin shock. Biochem and Biophys Res Commun.173:541-7.

Reeves, P.G., Nielsen, F.H., & Fahey, G.C. (1993). AIN-93 Purified diets for laboratory rodents: Final report of the American Institute of Nutrition Ad Hoc Writing Committee on the reformulation of the AIN-76A rodent diet. J Nutr. 123:1939-51.

Risler, N., Castro, C., Cruzado, M., Gonzalez, S., & Miatello, R. (2002). Early changes in proteoglycans production by resistance arteries smooth muscle cells of hypertensive rats. Am J Hypertens. 15: 416-21.

Rosen, O.M., & Lebwohl, D:E. (1988). Polylysine activates and alters the divalent cation requirements of the insulin receptor protein tyrosine kinase. F.E.B.S. Letters. 231:397-401.

Rubanyi, G.M., & Vanhoutte, P.M. (1986). Superoxide anions and hypoxia inactivate endothelium-derived relaxing factor. Am J Physiol. 250:H822-7.

Russo, G., Leopold, J.A., & Loscalzo, J. (2002). Vasoactive substances: Nitric oxide and endothelial dysfunction in atherosclerosis. Vascul Pharmacol. 38:259-69.

Salaices, M., Marin, J., Rico, M.L. & Gonzales, C. (1983). Effects of verapamil and manganese on the vasoconstriction responses to noradrenaline, serotinin and potassium in human and goat cerebral arteries. Biochem Pharmacol. 32:2711-4.

Salvemini, D., Currie, M.G., & Mollace, V. (1996). Nitric oxide-mediated cyclooxygenase activation. J Clin Invest. 97: 2562-8.

Scion Image-Release Beta 4.02 software (http://www.scioncorp.com).

Schuschke, D.A., Reed, M.W., Saari, J.T., & Miller, F.N. (1992). Copper deficiency alters vasodilation in the rat cremaster muscle microcirculation. J Nutr. 122:1547-52.

Shimokawa, H. (1999). Endothelial dysfunction: a novel therapeutic target: Primary endothelial dysfunction: atherosclerosis. J Mol Cell Cardiol. 31:23-37.

Soma, M., Izumi, Y., Watanabe, Y., & Kanmatsuse, K. (1996). A nitric oxide synthesis inhibitor decreased prostaglandin production in rat mesenteric vasculature. Prostaglandins. 51: 225–32.

Strott, C.A. (2002). Sulfonation and molecular action. Endocrine Rev. 23: 703-732.

Sullivan, L.J., & Briggs, A.H. (1968). Effects of manganese on the response of aortic strips to angiotensin and norepinephrine contractions. J Pharmacol Exp Ther. 161:205-9.

Taddei, S., Virdis, A., Mattei, P., & Salvetti, A. (1993). Vasodilation to acetylcholine in primary and secondary forms of human hypertension. Hypertension. 21:929-33.

Taddei, S., Virdis, A., Ghiadoni, L., Magagna, A., & Salvetti, A. (1997). Cyclooxygenase inhibition restores nitric oxide activity in essential hypertension. Hypertension. 29(Part2):274-9.

Tal, E., & Guggenheim, K. (1965). Effect of manganese on calcification of bone. Biochem J. 95:94-8.

Taylor, P.N., Patterson, H.H., & Klimis-Tavantzis, D.J. (1997). A fluorescence double-quenching study of native lipoproteins in an animal model of manganese deficiency. Biol Trace Elem Res. Oct-Nov:69-80.

Temple, N.J. (1983). Refined carbohydrates - A cause of suboptimal nutrient intake. Medical Hypotheses. 10:411-24.

Underwood, E.J. (1981). The incidence of trace element deficiency diseases. Phil. Trans. R. Soc. Lond. B. 294:3-8.

Van Hinsbergh, V.W., & Van Nieuw Amerongen, G.P. (2002). Intracellular signalling involved in modulating human endothelial barrier function. J Anat. 200:549-60.

Vanhoutte, P.M. (1981). Pharmacology of the blood vessel wall. J Cardiovasc Pharmacol. 3:1359-69.

Van Popele, N.M., Grobbee, D.E., Bots, M.L., Asmar, R., Topouchian, J., Reneman, R.S., Hoeks, A.P., Van der Kuip, D.A., Hofman, A., & Witteman, J.C. (2001). Association between arterial stiffness and atherosclerosis: The Rotterdam Study. Stroke. 32:454-60.

Vanhoutte, P.M. (1999). How to assess endothelial function in human blood vessels. J Hypertens. 17:1047-58.

Vanhoutte, P.M. (2003). Endothelial control of vasomotor function: from health to coronary disease. Circ J. 67:572-5.

Venema, R.C. (1999). Endothelial nitric oxide synthase regulation via protein protein interactions. In:Vascular Endothelium:Mechanisms of cell signaling. Catravas, J.D., Callow, A.D., & Ryan, U.S., Ed., IOS Press.

Vogel, R.A. (1997). Coronary risk factors, endothelial function and atherosclerosis: a Review. Clin Cardiol. 20:426-32.

Wambi-Kiesse, C.O., & Katusic, Z. (1999). Inhibition of copper/zinc superoxide dismutase impairs NO*-mediated endothelium-dependent relaxations. Am J Physiol Heart Circ Physiol. 276(3 Pt2):H1043-8.

Wang, W., & Diamond, S.L. (1997). Does elevated nitric oxide production enhance the release of prostacyclin from shear stressed aortic endothelial cells? Biochem Biophys Res Commun. 233:748–51.

Wang, W., Wang, S., Yan, L., Madara, P., Del Pilar Cintron, A., Wesley, R.A., & Danner, R.L. (2000). Superoxide production and reactive oxygen species signaling by endothelial nitric oxide synthase. J Biol Chem. 275:16899-903.

Weaver, J.A., Maddox, J.F., Cao, Y.Z., Mullarky, I.K., & Sordillo L.M. (2001). Increased 15-HPETE production decreases prostacyclin synthase activity during oxidant stress in aortic endothelial cells. Free Radical Biology & Medicine. 30:299–308.

Wedler, F.C. (1994). Biochemical and nutritional role of manganese: an overview. In: Manganese in Health and Disease (Klimis-Tavantzis, D.J., ed.) pp.2-37. CRC Press, Inc. Boca Raton, FL.

Weisiger, R.A., & Fridovich, I. (1973). Superoxide dismutase. Organelle specificity. J Biol Chem. 248:3582–92.

Weiss, G.B., & Kutsky, P. (1985). Effects of manganese on ^{45}Ca mobilization and contractile responses in rabbit aortic smooth muscle. Gen Pharmacol. 16(2): 97-102.

Williams, M.D., Van Remmen, H., Conrad, C.C., Huang, T.T., Epstein, C.J., & Richardson, A. (1998). Increased oxidative damage is correlated to altered mitochondrial function in heterozygous MnSOD Knockout mice. J Biol Chem. 273:28510-5.

Wolinsky, I., Klimis-Tavantzis, D.J., & Richards, L. (1994). Manganese and bone metabolism. In: Manganese in Health and Disease (Klimis-Tavantzis, D.J., ed.) pp.2-37. CRC Press, Inc. Boca Raton, FL.

Wu, G., & Morris, S.M. (1998). Arginine metabolism: nitric oxide and beyond. Biochem J. 336:1-17.

Yan, M., Liu, Z., Du, X.J., & Han, C. (1998). Effects of micromolar concentrations of Mn, Mo, and Si on alpha1-adrenoceptor-mediated contraction in porcine coronary artery. Biol Trace Elem Res. 64:75-87.

Yan, M., Liu, D.L., Chua, Y.L., Chen, C., & Lim, Y.L. (2001). Effects of micromolar concentrations of manganese, copper, and zinc on alpha1-adrenoceptor-mediating contraction in rat aorta. Biol Trace Elem Res. 82:159-66.

Yang, P., & Klimis-Tavantzis, D. (1998a). Effects of Dietary Manganese on Arterial Glycosaminoglycan Metabolism in Sprague-Dawley Rats. Biol Trace Elem Res. 64:275.

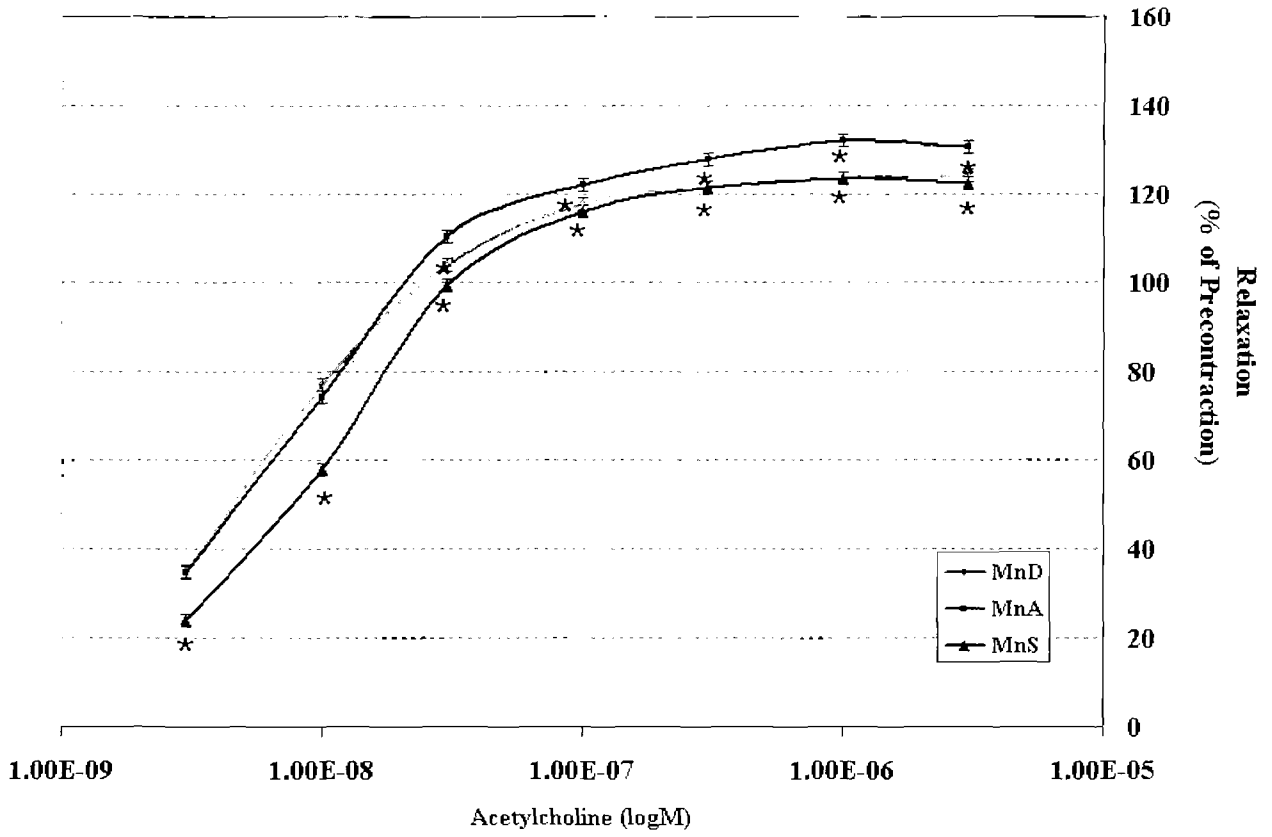
Yang, P., & Klimis-Tavantzis, D. (1998)b Manganese deficiency alters arterial glycosaminoglycan structure in Sprague-Dawley rat. J Nutr Biochem. 9:324-31.

Zanetti, M., Sato, J., Katusic, Z.S., & O'Brien, T. (2001). Gene transfer of superoxide dismutase isoforms reverses endothelial dysfunction in diabetic rabbit aorta. Am J Physiol Heart Circ Physiol. 280:H2516-23.

Zidenberg-Cherr, S., Keen, C., Lonnerdal, B., & Hurley, L. (1983). Superoxide dismutase activity and lipid peroxidation in the rat: developmental correlations affected by manganese deficiency. J Nutr. 113: 2498-504.

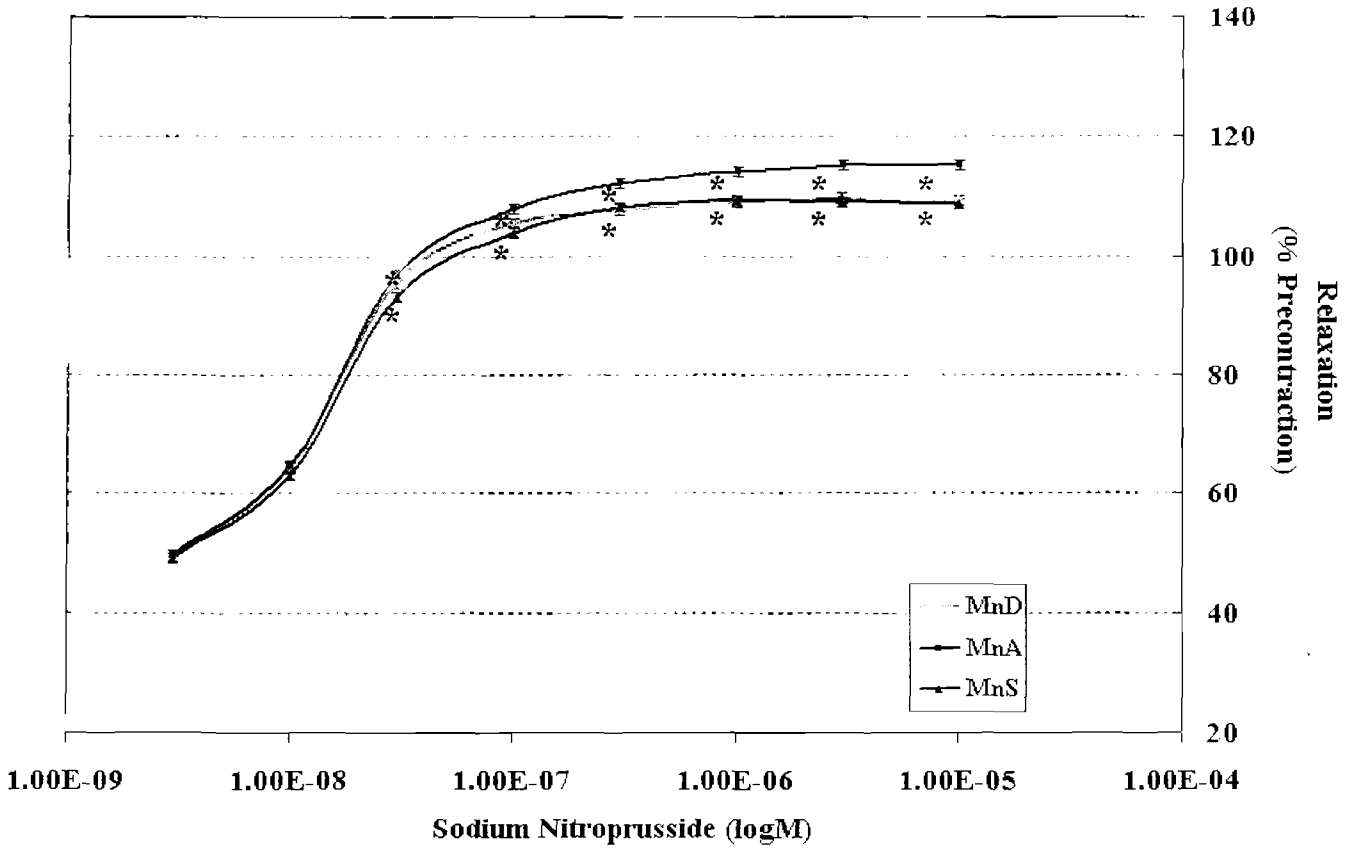
APPENDIX: Vasodilation Dose Response Curves

Figure A.1. Dose-response curves of the Ach-induced vasodilation of intact aortic rings among diet groups.



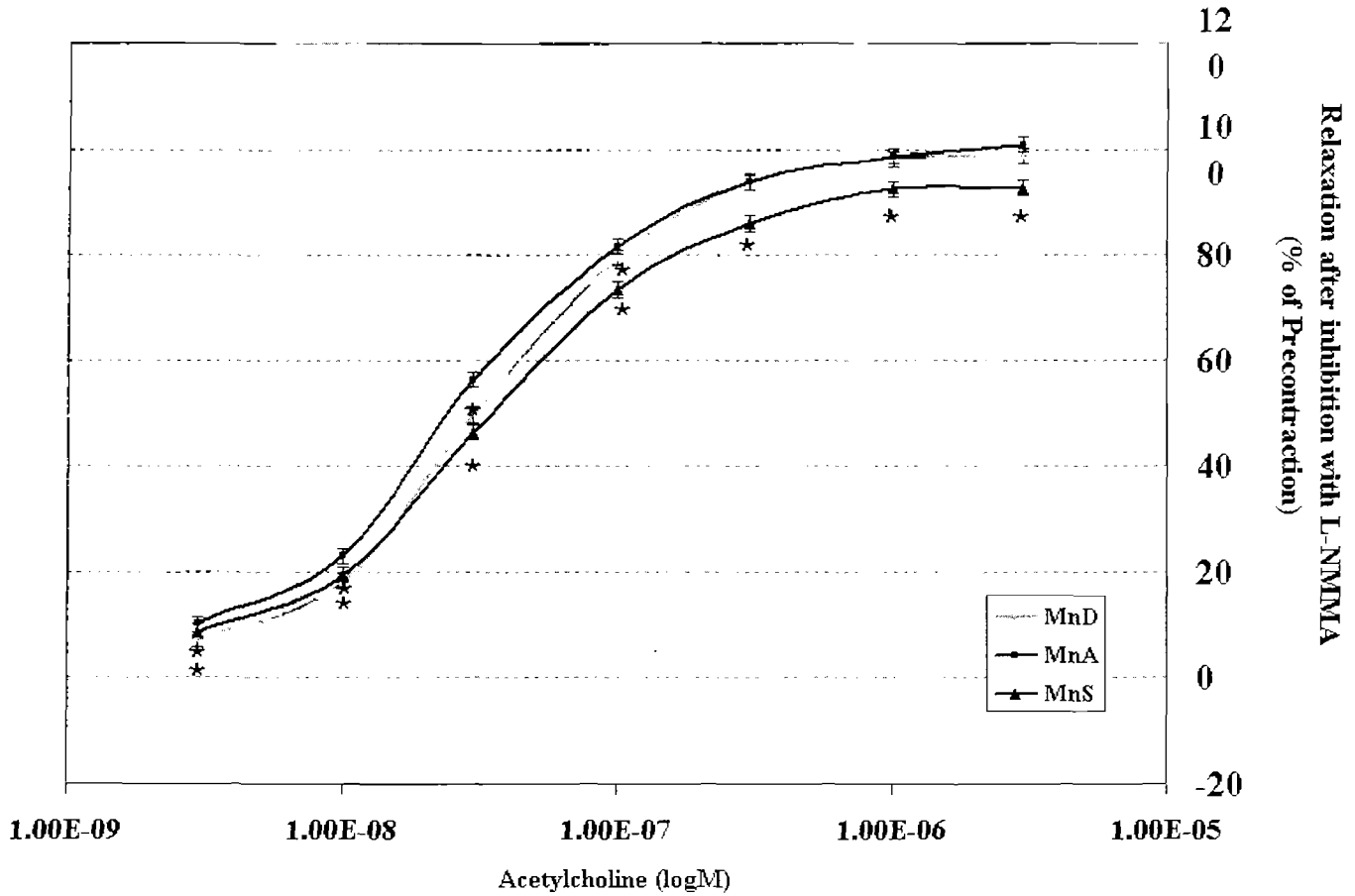
(*) Statistically significant at $p<0.05$ in comparison to MnA group

Figure A.2. Dose-response curves of the SNP-induced vasodilation of intact aortic rings among diet groups.



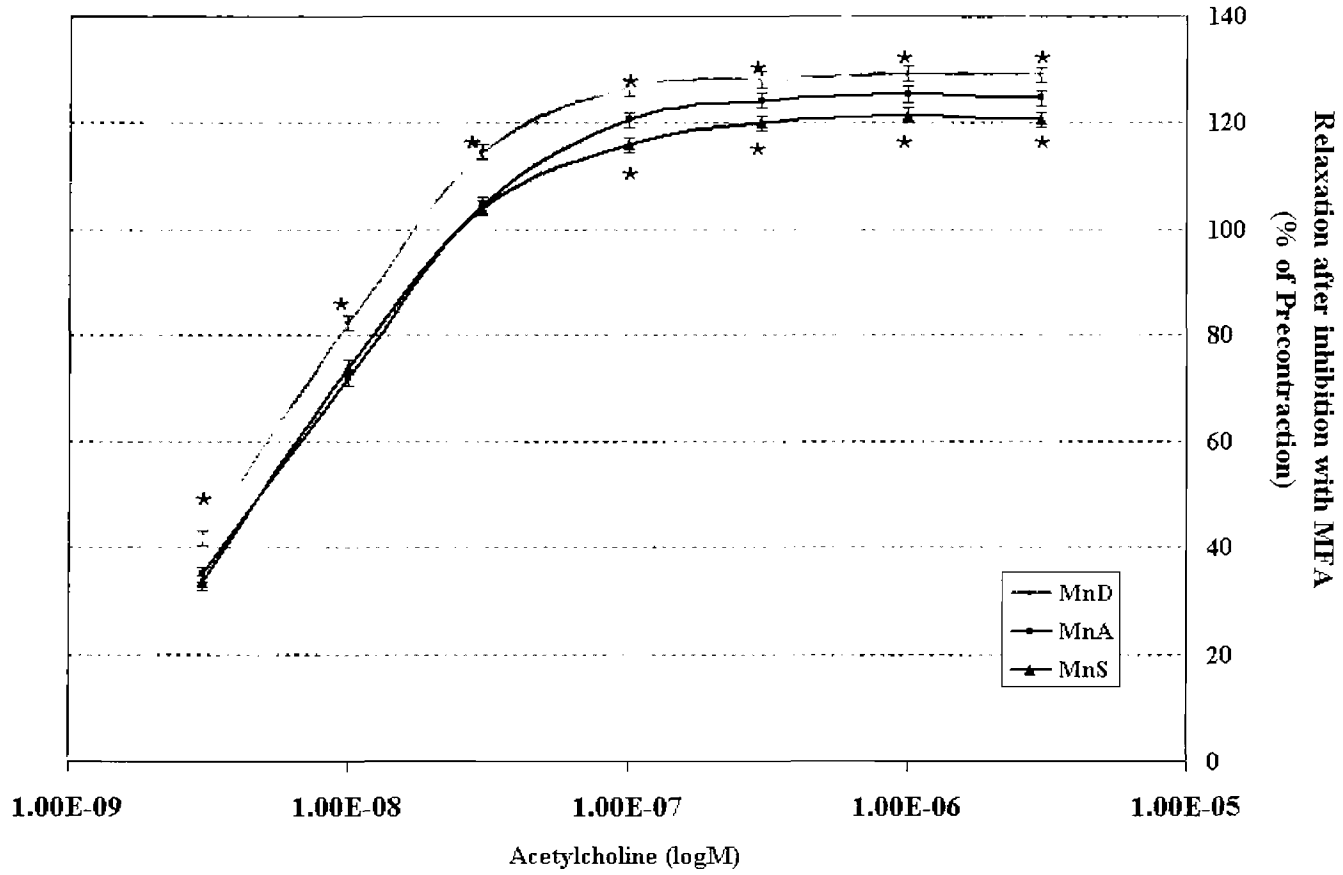
(*) Statistically significant at $p<0.05$ in comparison to MnA group

Figure A.3. Dose-response curves of the Ach-induced vasodilation of intact aortic rings among diet groups after NOS inhibition.



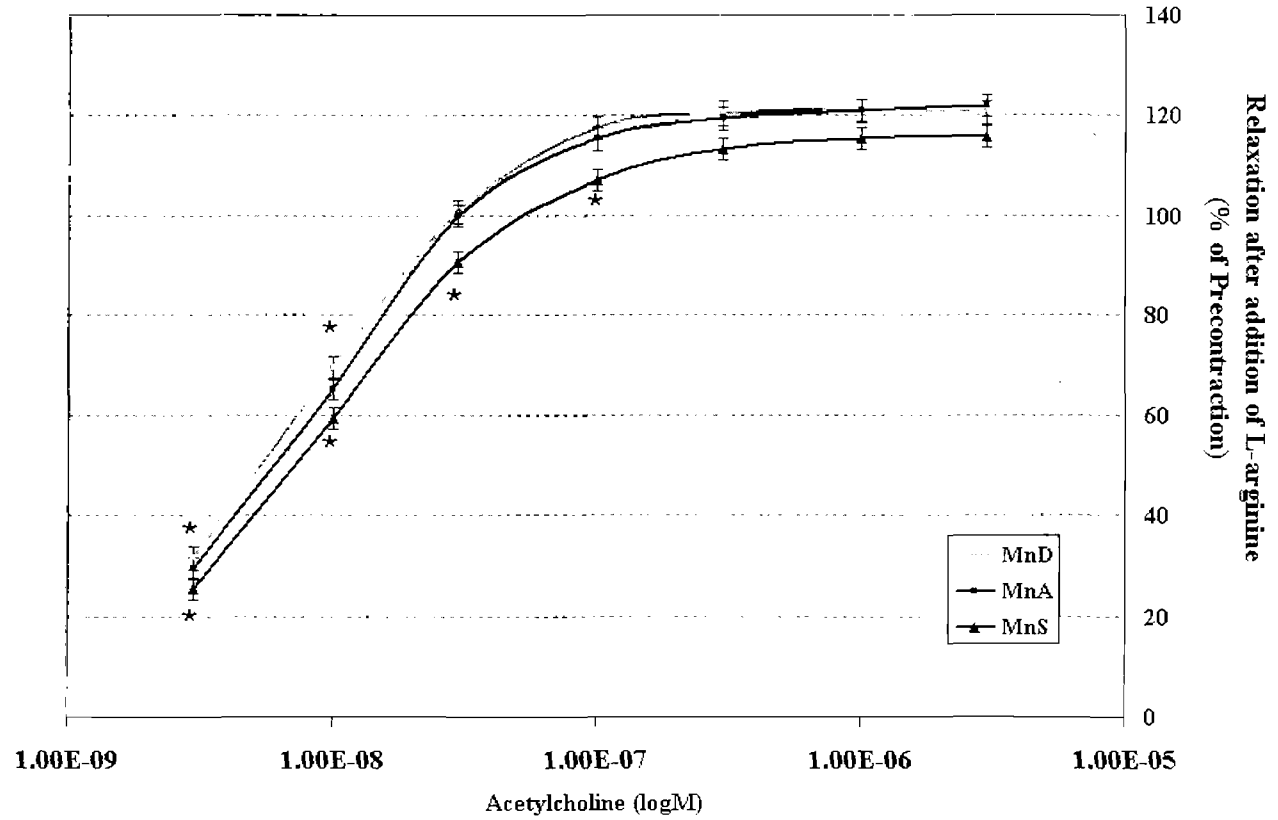
(*) Statistically significant at $p<0.05$ in comparison to MnA group

Figure A.4. Dose-response curves of the Ach-induced vasodilation of intact aortic rings among diet groups after COX inhibition.



(*) Statistically significant at $p<0.05$ in comparison to MnA group

Figure A.5. Dose-response curves of the Ach-induced vasodilation of intact aortic rings among diet groups after L-arginine supplementation.



(*) Statistically significant at $p<0.05$ in comparison to MnA group

BIOGRAPHY OF THE AUTHOR

Anastasia Z. Kalea was born in Kozani, Greece (Hellas) on December 1977. She was raised in Kozani and graduated from the 3rd General Education High School in 1995. She succeeded on the National Panhellenic Exams and entered Harokopio University, Athens, Greece in 1996. She graduated from Harokopio University in 2000 with a Bachelor's degree in Human Nutrition and Dietetics. She worked as a dietitian for six months conducting private practice and was involved in a national public health program before entering the Doctorate Program at the University of Maine in the Spring of 2001. After receiving her Ph.D. degree, Anastasia will be working as a Postdoctoral Fellow in the University of Maine. Anastasia would like to enter the Academia in order to teach and conduct research in the areas of Nutrition, Biochemistry and Physiology.

Anastasia is a candidate for the Doctor of Philosophy Degree in Food and Nutrition Sciences from The University of Maine in August, 2005.