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Dietary manganese affects the concentration, composition and sulfation pattern of heparan sulfate glycosaminoglycans in Sprague-Dawley rat aorta

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Abstract

We examined the effect of dietary Mn on the composition and structure of heparan sulfate (HS) glycosaminoglycans (GAGs) of rat aorta. Animals were randomly assigned to either a Mn deficient (MnD), adequate (MnA) or supplemented (MnS) diet (Mn < 1, 10–15 and 45–50 ppm, respectively). After 15 weeks, aortic tissue GAGs were isolated with papain digestion, alkaline borohydride treatment and anion-exchange chromatography. Cellulose acetate electrophoresis and treatment of the fractions with specific lyases revealed the presence of three GAG populations, i.e. hyaluronan (HA), heparan sulfate (HS) and galactosaminoglycans (GalAGs). Disaccharide composition of the HS fractions was determined by HPCE following treatment with heparin lyases I, II and III. In MnS aortas we observed increased concentration of total GalAGs and decreased concentration of HS and HA, when compared to MnA aortas. Aortas from MnD and MnA rats appeared to have similar distribution of individual GAGs. Heparan sulfate chains of MnS aortas contained higher (41%) concentration of non-sulfated units compared to MnA ones. Variable amounts of trisulfated and disulfated units were found only in MnD and MnA groups but not in MnS. Our results demonstrate that HS biosynthesis in the rat aorta undergoes marked structural modifications that depend upon dietary Mn intake. The reduced expression and undersulfation of HSPGs with Mn supplementation might indicate a reduced ability of vascular cells to interact with biologically active molecules such as growth factors. Alterations in cell-membrane binding ability to a variety of extracellular ligands might affect signal-transduction pathways and arterial functional properties.

Abbreviations: CS – chondroitin sulfate; CVD – cardiovascular disease; DS – dermatan sulfate; GAG(s) – glycosaminoglycan(s); GalAGs – galactosaminoglycans; HA – hyaluronic acid; HPCE – high performance capillary electrophoresis; HS – heparan sulfate; HSPGs – heparan sulfate proteoglycans; Mn – manganese; MnA – manganese adequate; MnD – manganese deficient; MnS – manganese supplemented; PG(s) – proteoglycan(s); UA – uronic acid.

Introduction

Several trace elements, such as zinc, copper, selenium, magnesium, manganese (Mn), nickel and molybdenum, have been reported to affect the process of cardiovascular disease (CVD) by altering the conformation of ligand-receptor binding and by interacting with ion channels, neurotransmitters, receptors and/or ionic channel-receptor complexes (Radhakrishnamurthy *et al.* 1989;

Klimis-Tavantzis *et al*. 1993; Kaji *et al*. 1994; Yang & Klimis-Tavantzis 1998a, b; Hennig *et al*. 1999; Nilius 2001; Dugan *et al*. 2003). The above interactions are influenced and regulated by the membrane macromolecular organization. One significant function of Mn is its effect on proteoglycan (PG) and glycosaminoglycan (GAG) metabolism (Leach 1971). Manganese functions as a specific activator of glycosyltransferases, enzymes involved in the elongation and polymerization of GAG chains in connective tissue (Leach *et al*. 1969). Manganese deficiency affects GAGs biosynthesis and results in decreases in total and individual GAGs, especially chondroitin sulfate (CS) in chick cartilage and rat skin (Leach *et al*. 1969; Bolze *et al*. 1985; Leach 1986; Shetlar & Shetlar 1994). Manganese also effectively activates sulfotransferases, enzymes involved in GAG sulfation and synthesis (Gundlach & Conrad 1985). We reported in the past that Mn affects arterial glycosaminoglycan (GAG) metabolism by altering the total proteoglycan (PG) concentration, as well as the molecular weight and sulfation pattern of CS in rat aortic tissue (Klimis-Tavantzis *et al*. 1993; Taylor *et al*. 1997; Yang & Klimis-Tavantzis 1998a, b). Additionally, scanning electron microscopy revealed significantly higher surface damage of the endothelial cell layer in MnD rats and transmission electron microscopy showed less dense extracellular matrix surrounding smooth muscle cells, especially in the medial layers of MnD rats (Ekanayake & Klimis-Tavantzis 1995; Yang & Klimis-Tavantzis 1998a, b).

Glycosaminoglycans are functionally important macromolecules of the arterial wall. With the exception of hyaluronan, they are covalently bound to proteins forming PG molecules. Chondroitin sulfate (CS), heparan sulfate (HS) and dermatan sulfate (DS) are the major PGs identified to exist in blood vessels (Wight 1989). Heparan sulfate is the most common GAG of the endothelial cell surface PGs. Heparan sulfate is composed of repeating disaccharide units containing one uronic acid (UA) and one D-glucosamine (GlcN) which might be either *N*-acetylated (GlcNAc), *N*-sulfonylated (GlcNS) or unsubstituted. Sulfate groups might also be esterified with hydroxyl groups. The structural diversity of HS chains enables their PGs (such as syndecans, fibroglycans, glypicans or perlecan) to interact in divergent ways with biologically effective molecules, such as enzymes, cytokines, growth factors and proteins. This new class of information-dense, high-specificity, multifunctional cell regulators plays a critical role in maintaining membrane fluidity and in affecting key events in the process of atherosclerosis (Wight *et al*. 1992), such as endothelial cell permeability (Hayward *et al*. 1997), monocyte and leukocyte adhesion (Wight *et al*. 1992; Woods *et al*. 1998), platelet aggregation and adhesion (Wight *et al*. 1992; Chon & Chaikof 2001) and vascular smooth muscle cell migration and proliferation (Wight *et al*. 1992; Kaji *et al*. 1994). Over the past decade our understanding of the functions of these molecules has shifted dramatically. We now know that disease conditions, such as vascular injury (Han *et al*. 1997) inflammation, atherosclerosis (Hoff & Wagner 1986; Theocharis *et al*. 1999; Theocharis *et al*. 2002) and hypertension (Risler *et al*. 2002) alter the expression and distribution of heparan sulfate proteoglycans (HSPGs), which might affect vascular responses.

Since there is limited information on the effect of Mn on rat aortic GAG structure, we determined and compared the overall GAG composition as well as its disaccharide composition and sulfation pattern of HS in aortas isolated from rats fed different concentrations of Mn.

Materials and methods

Chemicals

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 form from Sigma-Aldrich Chemical Co and were dissolved in distilled water. Papain 2xcrystallized, chondroitinases AC and ABC, as well as heparin lyases I and II were obtained from Sigma-Aldrich Chemical Co. DEAE-Sephacel was purchased from Pharmacia. Heparin lyase III were purchased from Seikagaku America. Standard preparations of differently sulfated D-disaccharides from heparin and heparan sulfate [ΔUA-(1 → 4)-GlcNAc: αΔdi-nonS, ΔUA-2S-(1 → 4)-GlcN: Δdi-mono2S, ΔUA-2S-(1 → 4)-GlcNAc: αΔdi-mono2S, ΔUA-(1 → 4)-GlcNS: Δdi-monoNS, ΔUA-2S-(1 → 4)-GlcN-6S: Δdi-di(2,6)S, ΔUA-(1 → 4)-GlcNS-6S: Δdi-di(6,N)S, ΔUA-2S-(1 → 4)-GlcNS: Δdi-di(2,N)S

and ΔUA-2S-(1 → 4)-GlcNS-6S: Δdi-tri(2,6,N)S], were also obtained from Sigma-Aldrich Chemical Co. All other chemicals used were of the best commercial available grade.

Animal care

Thirty weanling male Sprague-Dawley rats (Charles River Laboratories) were randomly assigned to three diet groups, a Mn deficient (MnD, Mn: < 1 ppm), Mn adequate (MnA, Mn: 10–15 ppm) or Mn supplemented (MnS, Mn: 45–50 ppm) of 10 rats each. 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 fourteen weeks (Klimis-Tavantzis *et al.* 1993; Taylor *et al.* 1997, Yang & Klimis-Tavantzis 1998a). About 10 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, our MnA diet group was our control group. Animals were individually housed in metal mesh-bottomed cages in an environmentally controlled room (maintained at 22 °C with a 12:12 h light: dark cycle) and were weighed weekly. The Animal Care and Use Committee of the University of Maine approved all animal care and experimental procedures.

Diets

Diets were mixed in our laboratory from purified ingredients, as described before (Klimis-Tavantzis *et al.* 1993; Taylor *et al.* 1997; Yang & Klimis-Tavantzis 1998a; Kalea *et al.* 2005). Vitamin (A.O.A.C. Special Vitamin Mixture, Harlan Tekland) and mineral mixes (ICN Biochemicals) were commercially prepared. Manganese content of the diets and tap water were determined by atomic absorption spectroscopy (detection limit 0.01 ppm) in Maine Forest and Agriculture Experiment Station Analytical Laboratory of the Plants and Soils Department of the University of Maine. The dietary concentration of Mn was tested every time following diet preparation and were stored at 4 °C for a maximum of 3–6 days following preparation. Food consumption was measured daily in all animal groups. Tap water (below the detection limit for Mn) and food was provided *ad libitum*.

Tissue sampling and preparation

At the end of the feeding period (14 weeks), food was withheld for 12–14 h. Animals were anesthetized in a chamber with 95% CO_2/ 5% O_2, for approximately 2 min. Thoracic aortas and livers were carefully removed from the animals and washed with PSS (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, freshly prepared) as previously described (Kalea *et al.* 2003, 2005; Norton *et al.* 2005). Livers were weighed, 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 of the Plants and Soils Department of the University of Maine.

The thoracic aorta from each rat was removed and submerged in a petri dish filled with PSS at room temperature. The aortic segment was cleaned of adherent fat and connective tissue and was stored at −70 °C. When all tissue specimens were available from all diet groups, aortas were powdered under liquid N_2 atmosphere. The powdered aortas were defatted twice with a methanol/chloroform solution (1:1, v/v) at 4°C for 16 h using 2×40 ml/g wet tissue (Theocharis *et al.* 1999). Tissue was rinsed with acetone and dried under vacuum.

Isolation of glycosaminoglycans

The powdered dry defatted tissues were rehydrated at 4 °C overnight with 0.1 mol/l sodium acetate, 2.4 mmol/l Na_2EDTA and 10 mmol/l cysteine hydrochloride (monohydrate). The rehydrated tissues were digested three times with 2×crystallized papain (2 mg papain/g of dry defatted tissue) for 20 h at 60 °C in a 0.1 mol/l sodium acetate buffer containing 2.4 mmol/l Na_2EDTA and 10 mmol/l cysteine hydrochloride (monohydrate) with frequent agitation (Theocharis *et al.* 1999, 2002). The digests were centrifuged at 4000×*g* for 10 min and the supernatants were collected and subjected to alkaline borohydride treatment using 1 mol/l $NaBH_4$ in 50 mmol/l NaOH. The mixture was heated at 45 °C for 48 h and then neutralized with 4 mol/l acetic acid. Aliquots derived from each aorta specimen were analyzed for uronic acid by the carbazole reaction as modified by Bitter and

Muir (Bitter & Muir 1962) and for sulfated GAGs by the dimethylmethylene blue assay (Farndale *et al*. 1986). No significant intrabatch differences were found in GAG content of aortas of all diet groups. Therefore, the samples containing the liberated GAGs from each animal group were pooled together and applied to a DEAE-Sephacel column (7×1.6 cm i.d.), eluted with 3 volumes of 0.1 M NaCl and with 10 volumes of a NaCl linear gradient ranging from 0.1 to 0.9 M. Fractions of 0.6 ml were collected and analyzed for uronic acid (Bitter & Muir 1962), where the absorbance of the end product at 530 nm is linearly associated with the UA concentration. A concentration-curve was obtained and the UA-positive fractions were pooled. The obtained populations were precipitated twice with 4 volumes of absolute ethanol at 4 °C overnight. The precipitants were obtained after centrifugation at 11 000×g for 15 min and dissolved in 2× distilled H_2O.

Cellulose acetate membrane electrophoresis

Glycosaminoglycans were identified by cellulose acetate membrane electrophoresis using a 0.1 mol/l pyridine – 0.1 mol/l formic acid (pH 3.1) running buffer at a constant current of 0.5 mA/cm of membrane width for 50 min (Karamanos 1992). Membranes were stained for 10 min with 0.5% w/v Toluidine Blue in 15% v/v aqueous methanol and destained by subsequent washings with water and scanned for viewing.

Degradation of GAGs with specific lyases

Disaccharide composition and sulfation pattern of GAGs were estimated following digestions with chondro-/dermato-lyases or with heparin lyases and analyses of digests with high performance capillary electrophoresis (HPCE) (Karamanos & Hjerpe 2001; Lamari *et al*. 2002). Digestions with chondroitinases AC and ABC were performed at 37 °C overnight in 0.1 mol/l sodium acetate – 0.1 mol/l Tris–HCl buffer, pH 7.3, using 0.01 units/10μg of UA. Digestions with heparin lyases I, II and III were performed at 37 °C overnight in 20 mM acetate buffer (pH 7.0) containing 1 μmol of calcium acetate and using 0.05 units from each heparin lyase per 25 μg of UA. All digestions were terminated by placing the digested samples for 1 min in 100 °C water bath. After centrifuging the samples at 11 000 g for 3 min, aliquots were taken for cellulose acetate electrophoresis to confirm depolymerization and for HPCE analysis.

High performance capillary electrophoresis (HPCE) analysis

The analysis was performed with a $HP^{3D}CE$ (Agilent Technologies, Waldbronn, Germany) instrument with a built-in diode array detector set at 232 nm for detection of migrated peaks. Resolution and analysis were carried out on an uncoated fused-silica capillary tube (50 μm i.d., 64.5 cm total length, 56 cm effective length) at 25 °C, using 50 mmol/l phosphate buffer, pH 3.5 for determination of HS-derived Δ-disaccharides (Karamanos *et al*. 1996; Militsopoulou *et al*. 2002) and 50 mmol/l phosphate buffer, pH 3.0, for the determination of HA/GalAG-derived Δ-disaccharides (Karamanos *et al*. 1994; Lamari *et al*. 1999), at 30 kV, as it has been described. The operating buffer was filtered through a 0.2 μm membrane filter. Samples were introduced using the pressure mode (50 mbar×5 s) at the cathodic end (reversed polarity), so that the Δ-disaccharides would migrate from the negative to the positive electrode by their electrophoretic mobility and against the electro-osmotic flow of the buffer. Before each run the capillary tube was washed with 0.1 mol/l NaOH for 1 min, 2× distilled water for 1 min and with the operating buffer for 5 min. Each peak in electropherograms of samples was identified by comparing its UV–vis spectrum with that obtained for standard Δ-disaccharides and by co-injection with the respective standard Δ-disaccharide. Quantitation of disaccharides content in samples was achieved using the linearity graphs obtained for standard mixtures of the twelve HS-derived Δ-disaccharides and the HP ChemStation software (Hewlett Packard).

Statistical analysis

Animal weights and hepatic Mn content were compared using a one-way analysis of variance (ANOVA). Weekly differences in food intake among diet groups were compared using Student's *t*-tests. Statistically significant differences in UA concentration were evaluated by Student's *t*-tests using the Microcal Origin Software (version 3.2).

A p-value level of 0.05 or less was considered as statistically significant.

Results

Animal growth

All animals fed a MnD, MnA or MnS diet gained weight (Table 1). The rate of growth in MnD animals was slower compared to the MnA and MnS rats and was statistically significant after four weeks on the diets. Liver weights (Table 1) and food intake (data not shown) were not statistically significant among the diet groups. MnD animals had a significantly lower body weight on the 14th week when compared to the MnA/ Controls ($p < 0.05$), while MnS was significantly heavier (Table 1). Manganese deficiency was confirmed by hepatic Mn concentration, which was significantly lower at MnD group when compared to MnA/ Control ($p < 0.0001$). Hepatic Mn concentration between MnA and MnS groups was similar (Table 1).

Dry aorta weight and uronic acid (UA) content

Dry aorta weights in the MnD rats were significantly lower from those of MnA rats ($p < 0.001$); MnA and MnS aortic weights were not different (Table 1). Tissue papain digests of each aorta specimen were analyzed for UA content (Table 1). Uronic acid concentration (μg of UA per g of dry defatted aorta) of MnS aortas was significantly lower ($p < 0.001$) as compared to MnA, while no significant differences between MnD and MnA groups were observed. Furthermore, there were no *intrabatch* differences in UA concentrations of aortas from each diet group. Thus, further analyses were performed on pooled aortas (one pooled sample was prepared from each group) due to the limited availability of tissue from each rat.

Type of glycosaminoglycans and quantitation

GAGs present in the rat aortas of the three different diet groups treated with papain and alkaline borohydride were fractionated by ion-exchange on DEAE-Sephacel. The column was eluted stepwise with 3 volumes of 0.1 mol/l NaCl and a NaCl linear gradient ranging from 0.1 mol/l to 0.9 mol/l NaCl (10 volumes). As shown in Figure 1, ion-exchange chromatography of aortas of all diet groups separated five UA positive populations. Populations I, II, III, IV and V were pooled (as indicated) and concentrated with alcohol precipitation. All isolated populations were electrophoresed on cellulose acetate membranes. The electrophoresis of populations revealed that populations I and II in all diet groups did not stain with Toluidine blue. The UA detected in these populations might be due to UA containing glycoproteins not related to any GAG type.

Population III migrated with a mobility identical to that of standard HA (Figure 2). Population IV migrated as standard HS and population V appeared as a broad band located between the migration positions of DS and CS (Figure 2). Electrophoresis of populations III, IV and V after treatment with chondroitinase ABC showed that populations III and V were completely degraded, whereas population IV was degraded only after treatment with a mixture of all heparin lyases. Populations III, IV and V were free from protein: this was assessed by measuring the absorbance of each fraction at 280 nm and analyzing each pooled population for protein using the method of Lowry *et al.* (1951).

Table 1. Animal growth characteristics (body weight and dry aorta weight), hepatic Mn concentration and uronic acid concentration in aortas of the three diet groups[a].

Dietary groups	Body weight (g)	Dry aorta weight (mg)	Hepatic Mn (μg/mg of dry liver weight)[b]	UA in aortas (μg/mg of dry aorta weight)[c]
MnD (<1 ppm)	452 ± 16*	15.1 ± 3.0*	1.52 ± 0.11*	3.33 ± 0.43
MnA (10–15 ppm)	489 ± 8	18.5 ± 2.3	7.09 ± 0.24	3.32 ± 0.10
MnS (45–50 ppm)	523 ± 9*	17.2 ± 2.0	7.60 ± 0.20	2.45 ± 0.50*

[a]Means ± SD of n = 10 rats per diet group. [b]μg of Mn per mg of freeze dried liver tissue. [c]μg of uronic acid per mg of dry and defatted aortic tissue. *Statistically significant at $p \leq 0.05$, when compared to MnA/Control diet group, as assessed by one-way ANOVA.

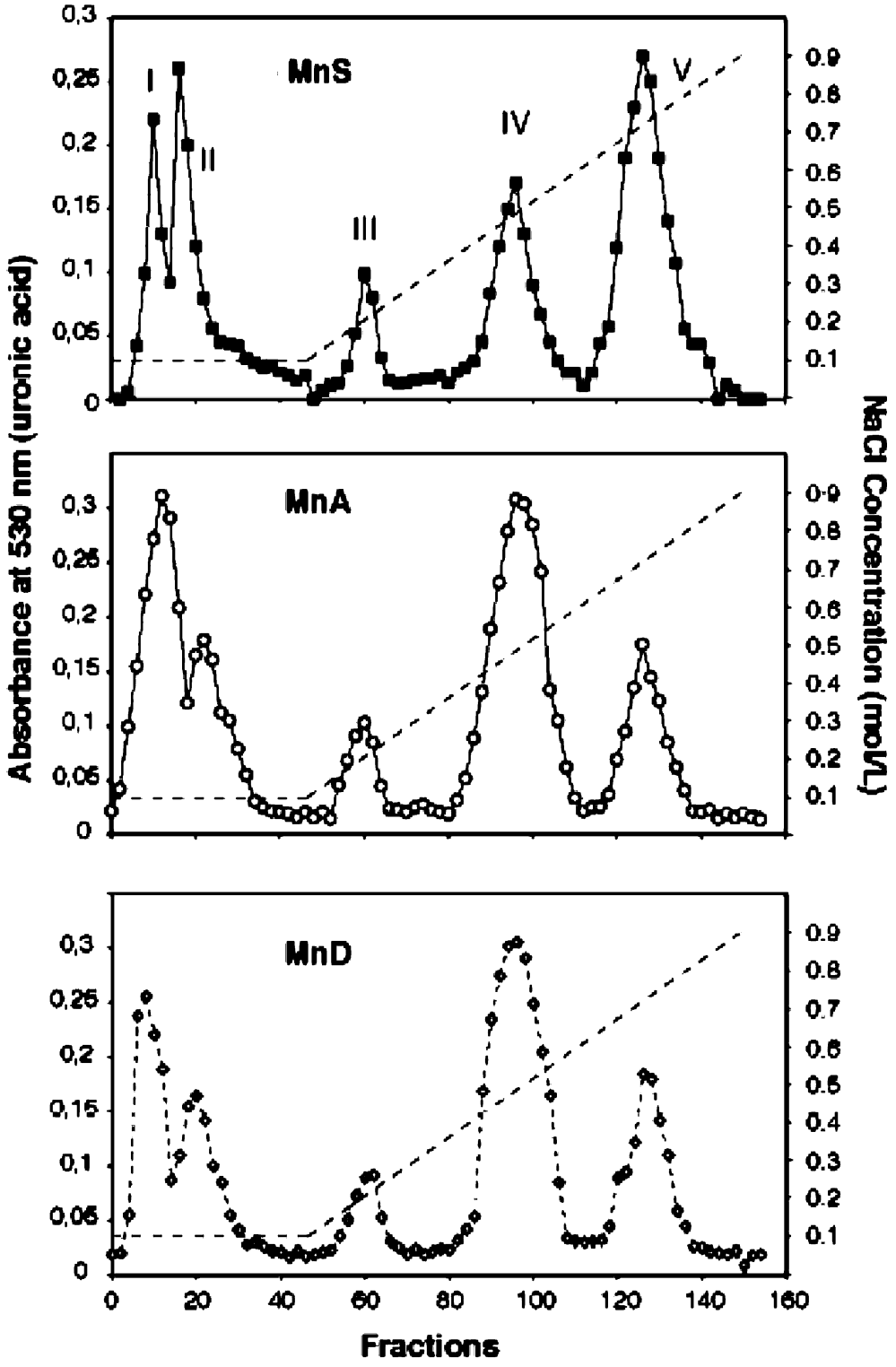

Figure 1. Fractionation of aortic GAGs from MnS (a), MnA (b) and MnD (c) rats by anion-exchange chromatography on DEAE-Sephacel column[a]. [a]The column was eluted stepwise with 0.1 mol/l NaCl (3 vol.) and a linear gradient ranging from 0.1 mol/l to 0.9 mol/l NaCl (10 vol.). Column recoveries were >90% (MnD, manganese deficient; MnA, manganese adequate; MnS, manganese supplemented; GAGs, glycosaminoglycans; I, II, III, IV, V: Fraction populations collected).

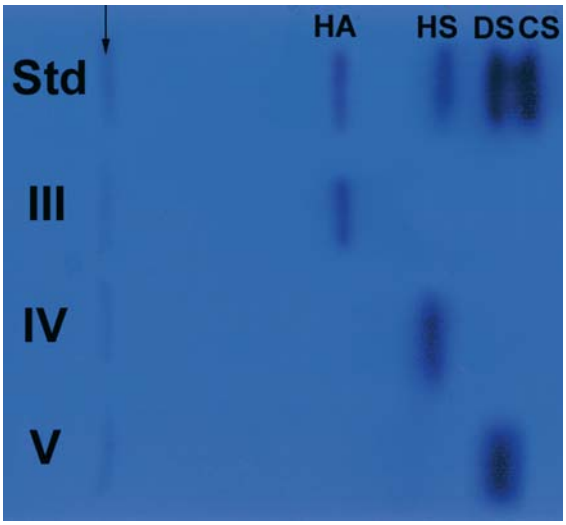

Figure 2. Cellulose acetate electrophoresis of the DEAE-Sephacel populations from aortas of MnS rats[a]. [a]Populations III, IV and V were electrophoresed on cellulose acetate membranes using 0.1 mol/l pyridine-0.1 mol/l formic acid (pH 3.1). Population III contains HA, population IV, HS and population V contains both CS and DS. The arrow shows the origin of electrophoresis (CS, chondroitin sulfate; DS, dermatan sulfate; HA, hyaluronic acid; HS, heparan sulfate ; Std, standard).

Identical chromatographic (Figure 1) and electrophoretic (Figure 2) profiles were obtained for aortas of all three diet groups. The chromatographic behavior and the electrophoretic profiles before and after treatment with specific lyases suggested that populations III, IV, and V represent HA, HS and CS/DS (GalAGs), respectively. The identity of population III was further examined following digestions of population III from all different dietary groups with chondroitinases ABC and AC and analysis by HPCE. This analysis confirmed that populations III contain only HA (only HA-derived non-sulfated Δ-disaccharides were detected). The amount of each GAG was quantified as UA content recovered from the column. HA of the MnA group (42 μg UA) accounts for 10.65% of the total GAGs recovered from the column. The respective percentage for HS of the MnA group was 64.2% and that for GalAGs was 25.1%. Marked quantitative differences between the MnA and the other two Mn dietary groups were observed (Table 2).

The distribution of GAGs in aortas (asμg of UA per g dry defatted tissue) from MnD, MnA and MnS rats is summarized in Table 2. According to the results obtained, MnS aortas contain primarily GalAGs, while HS corresponds to 34.5% of total GAGs. MnD and MnA aortas contain primarily HS and GalAGs constitute a smaller percentage of total GAGs (25–27.6%).

Disaccharide composition of glycosaminoglycans

Heparan sulfate preparations were digested with specific lyases and analyzed by HPCE to study their sulfation pattern. A mixture of heparin lyases I, II and III ensured almost complete digestion ($>92\%$) of HS chains to Δ-disaccharides, as proposed by Karamanos *et al.* (Karamanos *et al.* 1997; Karamanos & Hjerpe 2001). High-performance capillary electrophoresis analysis of the digests showed that αΔdi-nonS and αΔdi-mono2S account for approximately 90% of all HS-derived disaccharides (Table 3).

Heparan sulfate chains of MnD rats were similar to those of MnA rats (Table 3). In MnS rats the sulfation profile of HS chains was significantly different from MnA rats (Table 3). In particular, HS chains in MnS aortas had a 30% higher concentration of αΔdi-nonS, a 45–70% lower concentration of αΔdi-mono2S and the

Table 2. GAG-derived uronic acid concentration in each population isolated after anion-exchange chromatography on DEAE-Sephacel column in the three diet groups[a].

DEAE fraction populations	MnD	MnA	MnS
I & II (non-GAGs)	–	–	–
III (HA)	226 (9.1%)	207 (10.7%)	134 (8.6%)
IV (HS)	1574 (63.3%)	1249 (64.2%)	540 (34.5%)
V (GalAGs)	688 (27.6%)	488 (25.1%)	893 (57%)
Total GAGs	2488	1944	1567

[a]GAG-derived uronic acid concentration is expressed in μg of uronic acid per g of dry defatted tissue. Percentages of each type of GAG are given in parentheses.

Table 3. HS disaccharide composition in MnD, MnA and MnS aortas, determined by HPCE analyses after digestion with heparin lyases I, II and III[a].

Disaccharide	MnD	MnA	MnS
Δdi-tri(2,6,N)S	0.54 ± 0.02	0.83 ± 0.04	–
Δdi-di(2,N)S	4.00 ± 0.18	7.89 ± 0.39	–
Δdi-di(6,N)S	0.60 ± 0.03	1.12 ± 0.05	–
Δdi-di(2,6)S	0.33 ± 0.02	0.30 ± 0.02	–
Δdi-monoNS	2.85 ± 0.14	3.72 ± 0.15	2.30 ± 0.12
Δdi-mono2S	1.00 ± 0.04	1.66 ± 0.08	1.19 ± 0.05
αΔdi-mono2S	39.62 ± 1.61	34.71 ± 1.71	23.67 ± 1.08
αΔdi-nonS	51.06 ± 1.99	51.75 ± 2.26	72.83 ± 2.94

[a]Results are expressed as pmol/100 pmol of total recovered Δ-disaccharides and are the mean values of three separate determinations. (–): non-detectable disaccharides.

absence of di- and trisulfated Δ-disaccharides in comparison to those of rats in the control diet. Although HS chains from MnA and MnD rats had great similarities in their disaccharide composition, MnA aorta HS chains had higher concentration of in Δdi-di(2,N)S and Δdi-mo noNS when compared to MnD HS chains (Table 3).

Discussion

In this study, we present for the first time that dietary Mn affects both the composition and the sulfation profile of heparan sulfate GAGs in the Sprague-Dawley rat aorta. By using novel analytical techniques we confirmed the results of past studies (Yang & Klimis-Tavantzis 1998a, b) on the effect of dietary Mn on GAG concentration and structure in rat aorta.

In Mn deficiency, which was confirmed by lower hepatic Mn concentration, the distribution of the individual GAG fractions was similar to those of the MnA/Control group. The primary GAG in MnD aortic tissues was HS; the concentrations of CS and HA were similar between MnD and MnA aortas. The increased total UA concentration that appears in MnD aortas in comparison to MnA aortas is attributed to non-GAG fractions (fractions I & II in Table 2). The retardation of growth observed in MnD animals was independent of food intake. Retardation of rodent growth in Mn deficiency has also been reported by previous studies (Paynter 1980; Fahim *et al*. 1990; Klimis-Tavantzis *et al*. 1993; Taylor *et al*. 1997) and it seems to be an effect of reduced efficiency of food conversion with dietary Mn depletion (Yang & Klimis-Tavantzis 1998a).

When Mn was present at levels of supplementation, the concentration of GalAGs (CS/DS) was increased when compared to MnA/Control animals. The primary GAG component in MnS aortas was GalAGs and not HS. The concentration of HS in MnS aortas was almost half of the one in control aortas. In the past we reported a decrease in GalAGs concentration of MnD aortas, but the comparison was conducted between MnD and MnS aortas at the time, since 45–50 ppm was used as a control diet group (Yang & Klimis-Tavantzis 1998a, b). Manganese seems to be specifically required for optimal *in vivo* activity of the enzyme galactosyltransferase I, which participates in the biosynthesis of rat aortic GAGs (Yang & Klimis-Tavantzis 1998a). The increased concentration of GalAG chains in MnS animals might be attributed to an up-regulation of this Mn-dependent enzyme at concentrations of 45–50 ppm.

Analysis of sulfation pattern and disaccharide composition revealed similarities in the sulfation pattern and disaccharide profile of HS chains of MnD and MnA/Control groups. HS chains from MnD rat aortas had a slightly reduced concentration of Δdi-di(2,N)S and Δdi-monoNS when compared to MnA aortas. On the contrary, the HS sulfation pattern of MnS rat aortas appeared to be much different from the MnA one; MnS aortas had a higher concentration of non-sulfated Δ-disaccharides when compared to aortas of MnA/Control aortas. Additionally, di- and trisulfated Δ-disaccharides were not detected in the MnS HS structure, even though they were present in MnA rat aortas.

We have recently documented that dietary Mn affects the functional properties of the rat aorta by altering the responses of the contractile machinery to various agonists. Even though dietary Mn suppresses vascular sensitivity to an $\alpha 1$ adrenergic receptor agonist when present in the diet, only when it is present in 45–50 ppm (MnS diet) we observed a reduction in maximum vasoconstriction (in rats fed a MnD or MnA diet the responses of the contractile machinery were similar) (Kalea *et al*. 2005). These functional similarities between MnD and MnA aortas might well be in agreement with our observations on the similar GAG structures of MnD and MnA aortic vessels. Considering the fact that sulfation of the GAG chain is a biosynthetically regulated and a non-random process (Karamanos *et al*. 1995), the observed differences in sulfation patterns of HS chains might reflect modified biological roles of HSPGs of the aortic tissues of animals with different dietary Mn intake.

Heparin-like molecules play critical roles in the biochemical regulation of vascular repair (Hayward *et al*. 1997; Han *et al*. 1997). In the early stages of tissue injury and as a wound healing response, there is an enhanced accumulation of extracellular matrix, leukocyte adhesion, vascular smooth muscle cell (VSMC) proliferation and migration, which eventually lead to intimal thickening (Cizmeci-Smith *et al*. 1997; Koyama *et al*. 1998). Overexpression of HSPGs is an indicator of reconstitution of the normal endothelium during tissue injury (Bernfield *et al*. 1999; Nugent *et al*. 2000; Shriver *et al*. 2002; Kramer & Yost 2003). The expression of HSPGs is increased in carotid artery smooth muscle cells of male Sprague-Dawley rats in cases of vascular injury due to endothelial disruption, such as in balloon-catheter-induced injury (Cizmeci-Smith *et al*. 1997). Increased perlecan (extracellular HSPG) levels have also been reported in vascular injury of modified tissue-engineered cells (Nugent *et al*. 2000). In early atherosclerosis several factors such as neutrophils, lymphocytes, cytokines or even endotoxins might regulate the synthesis of HSPGs and facilitate this binding (Hayward *et al*. 1997; Li *et al*. 1997; Kolset & Salmivitra 1999). It has also been reported that pro-adhesive cytokines are more effective in recruiting leukocytes when cytokines are immobilized on the endothelial cell surface by endothelial cell HSPGs (Tanaka *et al*. 1993; Belting 2003; Gustafsson & Boren 2004). Thus, the decreased amount of HS in MnS diet, when compared to MnA might be to a certain extent due to decreased amounts of syndecan and/or glypican and support the hypothesis that Mn supplementation at 45–50 ppm may provide atheroprotection.

The composition of GAG chains as well as the structural alterations documented in HS chains might also be related to modified biological function in tissues undergoing repair processes. HSPGs such as perlecan and syndecan have been implicated in the internalization and lysosomal delivery of lipoproteins, either independently of receptors that facilitate the internalization or by affecting the lipoprotein receptor-related protein (LRP) (Williams & Tabas 1995; Li *et al*. 1997; Kunjathoor *et al*. 2002). In contrast to chondroitin sulfate proteoglycans (CSPGs), which play a role in LDL retention and modification in arterial intima (Pillarisetti 2000), HSPGs may act as potential receptors for atherogenic lipoproteins or facilitate the uptake of ligands by a process called ligand transfer to lipoprotein receptors, such as the LDL receptor-related protein (LRP), which binds and internalizes aggregated LDL in human vascular smooth muscle cells (Edwards *et al*. 1995; Albertini *et al*. 1997). Thus, HSPGs are important for the accumulation of cholesterol esters in fibroblasts (Kaplan *et al*. 1998). Additionally, an increase in sulfated GAGs rather than in non-sulfated, plays a critical role in enhancing blood vessel lipid deposition and accumulation (Li *et al*. 1997). Results from multiple studies suggest that the accumulation of sulfated PGs, such as perlecan in the vascular tissue might prolong the retention of lipoproteins in the vessel, increasing the potential for chemical modifications of these molecules such as oxidation, lipase modification or glycation (Tao *et al*. 1997; Llorente-Cortes *et al*. 2002). This response-to-retention process eventually leads to the aggregation, cellular uptake and lipid accumulation into macrophages and finally to the formation of foam cells in the intima. In our study, the decreased expression of HS in the MnS diet group seems to preserve endothelial function, indicating a cardioprotective effect of Mn in the dietary intake level (45–50 ppm) but not in the MnA level (10 ppm), which has been proposed by the American Institute of Nutrition (AIN) as the optimum dietary intake to attain normal animal growth in rats (Reeves *et al*. 1993).

In general, oversulfated disaccharide domains facilitate the binding of HSPGs with growth factors, matrix components, effectors and modulators of enzymatic catalysis (Lee *et al*. 2001). The biotransformation of carbohydrate chains by sulfonation is the recognized primary mechanism that influences biological activity by determining high specificity in these receptor-ligand binding interactions (Strott 2002; Militsopoulou *et al*. 2003). Suppression of the trisulfated disaccharide production in the animals supplemented with dietary Mn in levels 45–50 ppm, as well as the significant increase of the non-sulfated disaccharides in the same dietary group, suggests reduced binding ability of the aortic HSPGs to growth factors. Even though the physiological role of HSPGs as co-receptors is still not clear, it seems that overexpression of HSPGs increases vascular sensitivity, an indicator of the binding affinity of the ligand with the specific receptor, especially in low ligand concentrations (Lyon & Gallagher 1998; Kramer & Yost 2003). This is in accordance with our previous studies that demonstrated increased vascular sensitivity of the Mn deficient rat aortas to the α_1 adrenergic agonist L-Phenylephrine (Kalea *et al*. 2002, 2005).

The results of this study demonstrate that the biosynthesis of GAGs in the aorta of Sprague-Dawley rats undergoes marked modification, which depends upon the dietary concentration of Mn. Decreased HS concentration and undersulfation of HS chains with Mn supplementation (45–50 ppm) might alter cell binding to a variety of extracellular ligands and thus affect functional and signal transduction pathways of the aortic wall. Further studies are necessary to determine whether the above structural alterations observed in HSPGs with Mn supplementation, involve changes in biophysiological processes that are essential in arterial disease prevention, especially in high-risk populations.

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