Xanthine Oxidase and Cardiovascular Risk in Obese Children

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Abstract

Background: Pathological mechanisms of how childhood obesity leads to increased risk of cardiovascular disease (CVD) are not fully characterized. Oxidative-stress-related enzymes, such as xanthine oxidase (XO), have been linked to obesity, endothelial dysfunction, and CVD in adults, but little is known about this pathway in children. The aim of this study was to determine whether differential XO activity is associated with endothelial dysfunction, CVD risk factors, or cytokine levels.

Methods: Fasting plasma samples were obtained from obese (BMI $\geq$ 95th percentile; $n=20$) and age- and gender-matched healthy weight (BMI >5 th and <85 th percentile; $n=22$) children and adolescents (mean age, 12 ± 3 years) to quantify XO activity. In addition, fasting cholesterol, insulin, glucose, blood pressure, endothelial function, and cytokine levels were assessed.

Results: We observed a 3.8-fold increase in plasma XO activity in obese, compared to healthy weight, children (118 ± 21 vs. 31 ± 9 nU/mg of protein; $p < 0.001$). Plasma XO activity was correlated with BMI z-score ($r = 0.41$), waist circumference ($r = 0.41$), high-density lipoprotein cholesterol ($r = -0.32$), oxidized low-density lipoprotein ($r = 0.57$), adiponectin ($r = -0.53$), and monocyte chemoattractant protein-1 ($r = -0.59$).

Conclusion: XO activity is highly elevated in obese children and correlates with CVD risk factors, suggesting that XO may play a role in increasing cardiovascular risk early in life in the context of obesity.

Introduction

Obesity rates in the United States among children and adolescents ages 2–19 years old have risen nearly 3-fold since 1980, to a rate of nearly 17% in 2010.¹ A high rate of obesity in children has led to an increased cardiovascular disease (CVD) risk; for example, a population-based study showed that nearly 70% of obese children had at least one risk factor for CVD.² The pathological mechanisms of how childhood obesity leads to increased risk of CVD are not fully characterized. Oxidative stress (OS) is one factor that plays an important role in the cardiovascular system and controls endothelial function, vascular tone, and cardiac function. The major sources of vascular OS occur through generation of reactive oxygen species (ROS) by the enzymes, nicotinamide adenine dinucleotide phosphate oxidase, uncoupled nitric oxide (NO) synthase, and xanthine oxidase (XO).^{3,4} ROS generated from XO during production of uric acid, such as superoxide anion, are known to affect endothelial function by sequestering the endogenous vasodilator, NO. For example, NO-stimulated blood flow was increased and endothelial

dysfunction was reversed when XO was inhibited by allopurinol in adult smokers⁵ and patients with diabetes,⁶ which shows that XO plays a role in CVD in adults. However, little is known about how OS pathways, such as XO, influence cardiovascular risk (CVR) factors in children.⁷ The primary aim of this study was to determine whether plasma XO activity is increased in obese children, compared to healthy weight children. The secondary aim was to evaluate whether XO activity is associated with CVD risk factors in children by studying the correlations between XO activity and CVD risk factors and cytokine levels.

Methods

This was a cross-sectional study of 42 children and adolescents (mean age, 12 ± 3 years) from the Minneapolis-St. Paul metropolitan area who were categorized into two groups (healthy weight and obese), based on age- and sex-specific BMI percentiles.⁸ Data were collected between 2007 and 2012. Healthy weight subjects were frequency matched for age and gender with subjects in the obese group. Healthy weight subjects were classified as having a

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BMI >5th and <85th percentile ($n=22$) and participated in a study evaluating CVR factors among families. Obese subjects were classified as having a BMI ≥ 95 th BMI percentile ($n=20$) and were recruited from the University of Minnesota Amplatz Children's Hospital Pediatric Weight Management Clinic (Minneapolis, MN). Parental consent and child assent were obtained by the study coordinator before the study date. The protocol was approved by the University of Minnesota Institutional Review Board and was in accord with the ethical standards of the Helsinki Declaration of 1975, as revised in 2008. Height and weight were obtained using a standard stadiometer and electronic scale, respectively, with participants wearing light clothes and no shoes. BMI was calculated as weight in kilograms divided by height in meters squared. Waist circumference was measured mid-way between the base of the ribs and the superior iliac crest (to the nearest 0.5 cm), taken in duplicate, and the mean values were used in the analyses. Seated blood pressure was obtained after 5 minutes of quiet rest, on the right arm using an automatic sphygmomanometer. Three consecutive blood pressure measurements were taken with a 3-minute rest between. Blood pressure was then averaged. Blood samples were collected after a minimum 8-hour fast. Lipids, glucose, and insulin assays were conducted with standard procedures at the Fairview Diagnostic Laboratories, Fairview-University Medical Center (Minneapolis, MN), CDC-certified laboratory. Homeostasis model assessment of insulin resistance was calculated using methods previously described.⁹ Flow-mediated dilation (FMD) of the brachial artery was measured in a subset of the participants ($n=16$), as described previously.¹⁰ Cytokine and OS markers were measured using a Luminex multiplex bead array assay. Measurement of OS and inflammation blood markers in plasma included oxidized low-density lipoprotein (LDL; Mercodia, Winston-Salem, NC), C-reactive protein (CRP; Immundiagnostik AG, Bensheim, Germany), leptin, monocyte chemoattractant protein-1 (MCP-1), serpin E1, adiponectin, resistin, and factor D. Plasma was stored frozen at -70°C until assayed at the University of Minnesota Cytokine Reference Laboratory (Clinical Laboratory Improvement Amendments licensed) using enzyme-linked immunosorbent assay. Oxidized LDL, measured in this fashion, is a reflection of both minimally and fully oxidized LDL particles. The intra- and interassay coefficients of variation were as follows: oxidized LDL, 5.5–7.3 and 4.0–6.2, respectively; CRP, 5.5–6.0 and 11.6–13.8, respectively.

Plasma XO activity was determined by measuring the XO-specific conversion of lumazine to isoxantholumazine. Briefly, reaction mixtures containing 100 μL of plasma and 100 μL of 20 μM of lumazine in 50 mM of potassium phosphate buffer (pH, 7.4) were incubated at 37°C for 1 hour, followed by the addition of 200 μL of methanol (with umbelliferone as the internal standard) to stop the reaction and remove the proteins. After centrifugation and filtration, the supernatants were saved for high-performance liquid chromatography analysis of isoxantholumazine. The

supernatant (100 μL) was injected onto a 250-mm YMC-Pack Phenyl column (YMC Europe GmbH, Dinslaken, Germany), and fluorescence was measured on a Waters 474 Scanning Fluorescence Detector (Waters Corporation, Milford, MA) set at excitation wavelength of 340 nm and emission wavelength of 400 nm. One unit of XO activity is defined as the production of one micromole of uric acid per minute at 25°C . XO activity was normalized for total protein in the plasma samples.

Statistical Analyses

The Student's *t*-test was used to determine the differences between obese and healthy weight children in regard to plasma XO activity and baseline characteristics. Linear regression analysis was used to determine the association between plasma XO activity and CVR factors, cytokine levels, and OS levels. Data are presented as mean $\pm$ standard deviation, unless stated otherwise. Statistical significance was set at a *p* value ≤ 0.05 .

Results

Baseline characteristics are shown in Table 1. Compared to healthy weight children, obese children had higher systolic blood pressure (SBP; 116 ± 12 vs. 104 ± 10 mmHg; $p < 0.001$), total cholesterol (168 ± 25 vs. 150 ± 27 mg/dL; $p = 0.03$), LDL cholesterol (104 ± 20 vs. 85 ± 21 mg/dL; $p = 0.004$), triglycerides (113 ± 57 vs. 62 ± 21 mg/dL; $p < 0.001$), and insulin (13 ± 8 vs. 6 ± 5 mU/L; $p = 0.002$) and lower high-density lipoprotein (HDL) cholesterol (41 ± 9 vs. 53 ± 11 mg/dL; $p < 0.001$). XO was 3.8-fold higher in obese, when compared to healthy weight, children, as shown in Figure 1 (118 ± 21 vs. 31 ± 9 nU/mg of protein; $p < 0.001$; data are shown in mean $\pm$ standard error of the mean). FMD was not different between the obese and healthy weight children ($9.2 \pm 2.6\%$ vs. $10.5 \pm 5\%$). In a subset of subjects ($n=16$), plasma cytokine and adipokine levels were measured (Table 2). Compared to healthy weight children, obese children had higher leptin (53.3 ± 25 vs. 6.5 ± 5 ng/mL; $p < 0.01$) and lower MCP-1 (80 ± 42 vs. 289 ± 86 pg/mL; $p < 0.01$) and serpin E1 (16 ± 20 vs. 103 ± 22 ng/mL; $p < 0.01$) and a trend toward lower adiponectin (11.2 ± 3 vs. 16.5 ± 5 $\mu\text{g/mL}$; $p = 0.05$; Table 2). CVR factors that were positively associated with XO activity were BMI *z*-score ($R = 0.41$; $p < 0.01$), waist circumference ($R = 0.41$; $p < 0.01$), and oxidized LDL ($R = 0.57$; $p = 0.05$). CVD risk factors/adipokines that were negatively associated with XO activity were HDL ($R = -0.32$; $p = 0.04$), adiponectin ($R = -0.53$; $p = 0.04$), and MCP-1 ($R = -0.59$; $p = 0.02$; Table 3).

Discussion

Childhood obesity is associated with CVD risk factors, such as elevated SBP, LDL, triglycerides, and insulin as well as low HDL levels. In addition, obese children have adverse levels of cytokines/adipokines (*i.e.*, elevated leptin, CRP, and IL-6 and a reduction in adiponectin).^{11,12} In

Table 1. Baseline Characteristics

Characteristic	Weight classification		p value
	Lean (n=22)	Obese (n=20)	
Gender, females	36%	35%	0.93
Age, years	12±3	12±3	0.6
Height, cm	155±16	155±15	0.92
Weight, kg	48±16	85±32	<0.001
BMI, kg/m ²	19±3	33±8	<0.001
BMI z-score	0.28±0.7	2.4±0.4	<0.001
Waist circumference, cm	69±10	108±19	<0.001
Systolic blood pressure, mmHg	104±10	116±12	<0.001
Diastolic blood pressure, mmHg	57±10	61±10	0.07
Heart rate, beats/min	74±9	76±11	0.67
Total cholesterol, mg/dL	150±27	168±25	0.03
High-density lipoproteins, mg/dL	53±11	41±9	<0.001
Low-density lipoproteins, mg/dL	85±21	104±20	0.004
Triglycerides, mg/dL	62±21	113±57	<0.001
Glucose, mg/dL	86±7	85±6	0.24
Insulin, mU/L	6±5	13±8	0.002
Homeostasis model assessment of insulin resistance	1.4±1.1	2.8±1.8	0.004
Baseline brachial diameter, mm	3.6±0.8	3.2±0.3	0.24
Flow-mediated dilation, %	10.5±5	9.2±2.6	0.587

Data are presented as mean±standard deviation.

Figure 1. Plasma xanthine oxidase (XO) activity in healthy weight and obese children. Isoxantholumazine production from healthy weight ($n=22$) and obese ($n=20$) plasma samples standardized to purified XO standards of known activity. One unit of XO activity is defined as the production of one micromole of uric acid per minute at 25°C. Data are mean±standard error of the mean. * $p<0.001$, compared to the healthy weight group.

this study, we examined the association of childhood obesity and CVD factors on XO activity as a marker of OS. We directly measured XO activity in plasma by measuring the conversion of lumazine to isoxantholumazine, instead of measuring plasma uric acid levels, as an indirect method of quantitating XO activity. Using a direct method to measure XO is preferable because there are less-conflicting variables, as compared to using uric acid levels, which can change based on differences in diet and the ability to eliminate uric acid through the kidneys. It is known that hyperuricemia and gout are associated with obesity, but the cause of this high level of uric acid is not entirely clear. Elevated uric acid levels could be a result of decreased elimination of uric acid by the kidneys in an obese population or it could be a result of increased production of uric acid by XO. In our study, we observed that XO activity was significantly increased in obese children and adolescents (3.8-fold increase in XO activity) and that this XO activity was significantly associated with CVD risk factors, such as BMI z-score, waist circumference, HDL, and oxidized LDL and with the cytokines, adiponectin and MCP-1. These findings demonstrate that there is an increased production of uric acid in obese

Table 2. Baseline Cytokine and Oxidative Stress Marker Levels

	Weight classification		p value
	Lean (n=7)	Obese (n=9)	
Leptin, ng/mL	6.5 ± 5	53.3 ± 25	<0.01
Monocyte chemotactic protein-1, pg/mL	289 ± 86	80 ± 42	<0.01
Serpin EI, ng/mL	103 ± 22	16 ± 20	<0.01
Adiponectin, µg/mL	16.5 ± 5	11.2 ± 3	0.05
C-reactive protein, mg/L	0.4 ± 0.55	4.89 ± 6.12	0.06
Oxidized low-density lipoproteins ^a , U/L	76 ± 19	97 ± 30	0.18
Resistin, ng/mL	8.0 ± 4	6.8 ± 3	0.51
Factor D, ng/mL	2.4 ± 0.5	2.3 ± 0.4	0.94

Data are presented as mean ± standard deviation.

^aLean (n=5) and for obese (n=7).

Table 3. Correlations Between Xanthine Oxidase Activity and Oxidative Stress Markers and Cytokines

Cardiovascular disease risk factor or cytokine	R	p value
BMI (n=42)	0.26	0.10
BMI z-score (n=42)	0.41	<0.01
Waist circumference (n=42)	0.41	<0.01
Systolic blood pressure (n=42)	0.13	0.41
Diastolic blood pressure (n=42)	0.02	0.88
Heart rate (n=17)	0.45	0.07
Total cholesterol (n=41)	0.04	0.82
High-density lipoproteins (n=41)	-0.32	0.04
Low-density lipoproteins (n=41)	0.08	0.60
Triglycerides (n=41)	0.29	0.07
Glucose (n=41)	-0.18	0.27
Insulin (n=39)	-0.04	0.78
Homeostatis model assessment of insulin resistance (n=39)	0.06	0.71
Baseline brachial diameter (n=17)	-0.18	0.49
Flow-mediated dilation (n=16)	-0.14	0.61
Oxidized low-density lipoproteins (n=12)	0.57	0.05
C-reactive protein (n=16)	0.07	0.78
Adiponectin (n=16)	-0.53	0.04
Factor D (n=16)	0.27	0.31
Leptin (n=16)	0.29	0.27
Monocyte chemotactic protein-1 (n=16)	-0.59	0.02
Serpin EI (n=16)	-0.47	0.06
Resistin (n=16)	-0.31	0.25

children. In addition, the findings are also consistent with previous studies in an obese pediatric population whereby *in vivo* XO activity was increased, as assessed by *in vivo* caffeine phenotyping strategies¹³; and high serum uric acid concentrations were associated with metabolic syndrome and CVRs in similar pediatric populations.^{14–16} We observed that adiponectin was inversely associated with XO activity. This finding is similar to previous studies whereby plasma uric acid concentration (a surrogate for XO activity) was inversely proportional to adiponectin concentrations.^{17–20} Surprisingly, we observed that MCP-1 activity was inversely associated with XO activity (Table 3). *In vitro* studies in whole blood and in mononuclear cells demonstrated that MCP-1 is up-regulated in the presence of reactive oxidative intermediates that are produced by XO.²¹ Thus, one would anticipate that higher XO enzyme activity would lead to more ROS available to increase MCP-1 expression. A limitation of our study is that we were unable to determine serum uric acid concentrations or plasma NO concentrations resulting from limited samples and analyte instability. NO, unlike XO, is a negative regulator of MCP-1 expression. Codoner-Franch and colleagues observed that NO production is increased in obese children, which is contrary to what is observed in obese adults.²² It is plausible that the obese children in our population have elevated NO levels, which would serve as a better surrogate for MCP-1 expression.

Surprisingly, there was no correlation of FMD or SBP with XO activity, and this could be the result of elevated NO levels whereby elevated NO levels in obese children may counteract the increase in ROS resulting from increased XO activity and lead to a net no change in dilation of the arteries. An alternative explanation for the lack of correlation between FMD and XO activity is that baseline

uric acid levels may have more of an effect on FMD and SBP than XO activity. For example, clinical studies in adults have demonstrated that when treated with the XO inhibitor, allopurinol, chronic heart failure patients and type II diabetic patients with mild hypertension showed improvements in endothelial function.^{6,23} Even though these studies demonstrated that XO inhibition led to improvements in endothelial function, the role of uric acid and ROS in modulating endothelial function could not be separated because both uric acid levels and ROS generated by XO were reduced. However, contrary to the XO- and FMD-positive correlative findings, Doehner and colleagues only observed allopurinol improvement of endothelial function in patients with elevated uric acid with chronic heart failure,²⁴ thus, demonstrating that there are multiple components in addition to OS that contribute to the CVR factor.

In our study, we observed that HDL concentrations were inversely associated with XO activity. *In vitro* studies by Chander and Kapoor demonstrated that HDL can inhibit XO activity by 43% in an *in vitro* rat liver microsomal system. Thus, the higher the HDL, the lower the XO activity, which would decrease the generation of ROS associated with this enzyme.²⁵ This inhibitory effect of HDL on XO activity could mean that future studies looking at XO activity should control for HDL levels. A positive correlation was observed between XO activity and oxidized LDL in our study, and this was expected because ROS produced by XO are known to be sources of oxidation for LDLs. Oxidized LDL is elevated in severe pediatric obesity^{11,26} and is associated with CVR and insulin resistance in children and adolescents.^{10–12} This positive correlation between XO activity and oxidized LDL indicates that XO may play a role in atherosclerosis risk in childhood obesity because oxidized LDLs penetrate the endothelium and form plaques.^{27,28}

Conclusion

In conclusion, plasma XO activity in the obese group was increased by 3.8-fold, as compared to the healthy weight group, and was correlated with the CVD risk factors BMI z-score, waist circumference, HDL, and oxidized LDL and with the cytokines adiponectin and MCP-1. Results of this study provide evidence of a substantial increase in plasma XO activity in obese children, which could be a factor contributing to increased uric acid and CVD risk in the context of pediatric obesity. The results of this study could provide a basis for targeting and inhibiting XO as a measure for preventing CVD in obese children and adolescents. Further studies are needed to confirm these findings and to see whether inhibiting XO in childhood obesity decreases CVD risk.

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Author Disclosure Statement

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