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Urinary Catalytic Iron in Obesity

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INTRODUCTION: Obesity precedes the development of many cardiovascular disease risk factors, including type 2 diabetes mellitus (DM), hypertension, and chronic kidney disease. Catalytic iron, which has been associated with these chronic diseases, may be one of the links between obesity and these multifactorial diverse disorders.

OBJECTIVE: We investigated whether urinary catalytic iron is increased in obese individuals without DM and overt kidney disease.

STUDY DESIGN: We measured urinary catalytic iron using established methods in 200 randomly selected individuals without DM [100 who were obese (body mass index $\geq$30 kg/m^2) and 100 who were nonobese (body mass index $\leq$27)]. Participants were selected from an outpatient clinic and community setting and were part of an ongoing cross-sectional study of obesity in individuals between the ages of 18 and 70 years.

RESULTS: There was a significant difference in mean (95% CI) urinary catalytic iron excretion between the obese participants and the nonobese participants, 463 (343–582) nmol/mg [52.3 (38.8–65.8) nmol/μmol] vs 197 (141–253) nmol/mg [22.3 (15.9–28.6) nmol/μmol]; $P < 0.001$. The significant predictors of increased urinary catalytic iron were obesity ($P = 0.001$) and waist-to-hip ratio ($P = 0.03$).

CONCLUSIONS: Our study results demonstrate that obesity and waist-to-hip ratio are associated with increased urinary catalytic iron, which may be a useful marker of oxidative stress. Additional studies are needed to determine the role of catalytic iron in increased cardiovascular disease and chronic kidney disease associated with obesity.

Currently more than 400 million adults worldwide are obese *(1, 2)*, and obesity is linked to the growing prevalence of type 2 diabetes mellitus (DM) and cardiovascular disease (CVD) *(1)*. Results of several studies suggest a link between obesity and chronic kidney disease (CKD) *(3–7)*. There is a close association between CVD, CKD, and DM *(8)*. It is thus conceivable that a common-path physiological mechanism exists that links obesity, DM, CVD, and CKD.[5]

Catalytic iron, also known as labile iron, consists of chemical forms that can participate in redox cycling. This property makes iron potentially hazardous by enabling it to participate in the generation of reactive iron-oxygen complexes such as ferryl and perferryl ion and powerful oxidant species such as hydroxyl radical produced by the metal-catalyzed Haber-Weiss reaction *(9)*:

$$Fe^{3+} + O_2 \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^\bullet$$

$$O_2 + H_2O_2 \rightarrow O_2 + OH^- + OH^\bullet$$

Our bodies contain as much as 3–5 g of total iron, but the pool of labile iron that can be measured *(10)* is estimated to be <70–90 mg. Although a protective effect of iron chelators in animal models of cardiovascular and kidney disease was demonstrated more than 2 decades ago, the importance of labile iron in several disease states as well as potential sources of labile iron are only now beginning to be recognized.

There are 2 broad lines of evidence for the role of labile iron in different disease states: that it is increased, and that iron chelators provide a protective effect, thus supporting a cause-effect relationship. Such evidence has been reported in a variety of diseases including acute and chronic kidney disease *(11, 12)*, CVD *(13–15)*, and neurodegenerative disorders *(16, 17)*. Labile iron therefore seems to be a common link of cellular injury. We have postulated that labile iron is the

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[5] Nonstandard abbreviations: DM, type 2 diabetes mellitus; CVD, cardiovascular disease; CKD, chronic kidney disease; UMACR, urine microalbumin/creatinine ratio; BDI, bleomycin-detectable iron; UBDI/Cr, urinary BDI/creatinine ratio; WHR, waist-to-hip ratio.

common-path physiological link that ties together the disorders of obesity, DM *(18)*, CVD, and CKD. We have previously reported an increase of catalytic iron in patients with diabetic nephropathy *(11)* and in patients with other forms of CKD. The purpose of this study was to test the hypothesis that urinary catalytic iron is increased in obese patients who are without overt renal impairment and DM.

Research Design and Methods

PARTICIPANTS

In a random, stratified manner we selected 200 individuals between the ages of 18 and 70 years (100 obese and 100 nonobese; 50 men and women in each group). The study participants were selected from outpatient clinic and community settings and were participants in an ongoing cross-sectional study to investigate the endocannabinoid system in obesity. Our participant population groups were defined as follows: BMI $\leq$27 kg/m^2 (control nonobese group) and $\geq$30 kg/m^2 (obese group). For the purpose of this substudy examining catalytic iron, we excluded individuals who were pregnant or had DM or an estimated glomerular filtration rate $<$60 mL/min. The diagnosis of DM for exclusion purposes was made on the basis of medical history of such a diagnosis, use of DM medications, or fasting blood glucose concentration of $>$126 mg/dL ($>$7.0 mmol/L).

The study protocol was approved by an institutional review board, and all study participants gave informed consent. The participants underwent a history and physical examination and submitted blood while in a fasting state. During the study participant's clinic visit for the research study a random fasting spot urine sample was collected and kept in ice until it was divided into aliquots which were put into cryo-vials (within 30 min). These samples were snap frozen immediately and stored at −70°C for additional analysis. One patient in the control group and 3 patients in the obese group had known coronary heart disease, according to data from the patient history. A detailed history of each patient's medications, including over-the-counter medications, was obtained. One individual in each group was on an iron supplement, and 13 individuals in each group took multivitamins on a daily basis. One patient in the obese group and 2 patients in the control group stated that they took vitamin C. Nonsteroidal antiinflammatory drug use was reported by 11 patients in the obese group and 8 patients in the control group. Five patients in the control group and 5 patients in the obese group had urine microalbumin/creatinine ratios (UMACR) $>$30. There were 45 women who were postmenopausal (13 in the control group, and 32 in the obese group). None of the obese postmenopausal women were on hormone replacement therapy. In the control group, 12 postmenopausal women were on hormone replacement therapy. There were 4 individuals in the control group and 12 individuals in the obese group who were on lipid-lowering agents. The characteristics of the control and the obese groups are shown in Table 1.

URINARY CATALYTIC IRON TEST

The bleomycin-detectable iron (BDI) assay is based on the observation that the antitumor antibiotic bleomycin, in the presence of iron salt and a suitable reducing agent, binds to and degrades DNA, with the formation of a product that reacts with thiobarbituric acid to form a chromogen. The assay detects iron complexes capable of catalyzing free-radical reactions in biological samples. We tested for the reproducibility and intra- and interassay variability and found them to be acceptable according to standards, as described previously *(19–21)*. For the present study, the within-run and total SDs of the modified urinary BDI assay were determined by following the analytical evaluation method of Linnet and Boyd *(22)*. Two replicates per specimen per run, and 2 runs per day for 20 days were performed for the analysis, as described in CLSI EP5-T *(23)*. For measurement of urinary BDI in the middle range, the intraassay CV was 1.46% and the interassay CV was 19.25%. The initial measurement of catalytic iron was made in micromoles per liter. Then it was adjusted for urine creatinine concentration, and the results were expressed as the ratio of urinary BDI to creatinine (UBDI/Cr) in units of nanomoles per milligram of urine creatinine. The urine albumin was measured by use of the Nyco Card (Axis Shield) kit and expressed as micrograms per milligram of urine creatinine. We used the CR-S synchron (Beckman Coulter) creatinine estimation kit for the measurement of urine creatinine by the Jaffe kinetic method. The analysis was done in a Synchron CX5 Pro Beckman Coulter fully automated clinical chemistry analyzer, and the measured creatinine was expressed in milligrams per deciliter. Plasma glucose was measured by spectrophotometry. Plasma ferritin was measured by the clinical laboratory at the Central Arkansas Veterans Healthcare System using a 2-site immunoenzymatic sandwich assay with a Beckman-Coulter Unicell DXI 800.

MAIN OUTCOMES AND MEASURES

Urinary microalbumin, urinary creatinine, plasma ferritin, and total urinary catalytic iron (BDI) were measured, and UMACR and UBDI/Cr were calculated.

STATISTICAL ANALYSIS

Descriptive statistics for numerical variables are provided. Unpaired Student *t*-tests were performed for

Table 1. Characteristics of the participants in the 2 groups.[a]

	Controls (n = 100)	Obese individuals (n = 100)	*P*
BMI	22.9 (2.5)	38.5 (6.5)	<0.001
WHR	0.8 (0.1)	0.9 (0.1)	<0.001
Waist circumference, inches	31.2 (5.5)	44.7 (6.2)	<0.001
Age, y	37.4 (13.7)	43.9 (12.6)	0.006
Glucose, mg/dL[b]	65.7 (2.5)	79.3 (24.2)	<0.001
Systolic blood pressure, mm Hg	117.5 (13.0)	126.7 (15.9)	<0.001
Diastolic blood pressure, mm Hg	72.1 (10.6)	80.4 (10.7)	<0.001
Total cholesterol, mg/dL	182.2 (37.9)	186.1 (34.8)	0.46
LDL, mg/dL	100.6 (31.6)	111.6 (31.21)	0.02
HDL, mg/dL	65.7 (17.7)	51.6 (14.1)	<0.001
Triglycerides, mg/dL	84.5 (70.4)	118.1 (68.3)	0.001
Serum creatinine, mg/dL	0.9 (0.4)	0.8 (0.3)	0.32
Log UBDI/Cr	3.5 (2.5)	4.9 (2.2)	<0.001
Plasma ferritin, μg/L	63.6 (64.6)	80.4 (85.6)	0.12
UMACR, μg/mg	11.5 (13.0)	10.7 (10.4)	0.34
Log UMACR	2.1 (0.8)	2.1 (0.9)	0.78
UBDI/Cr in individuals with UMACR >30 nm/mg	203.4 (231.3)	333.4 (283.6)	0.47

[a] Results are expressed as mean (SD).
[b] To convert concentrations to millimoles per liter, multiply by 0.0555 for glucose; by 0.0259 for cholesterol, LDL, and HDL; by 0.0113 for triglycerides; and by 0.0884 for creatinine.

comparisons of mean values between any 2 groups. We used χ^2 tests and, when necessary, Fisher exact tests for testing differences in frequencies of categorical variables between any 2 or more groups. Pearson correlation coefficients were calculated for associations between 2 numerical measures. Ordinary multiple linear regression models were fitted to assess associations between the main outcome measures and various covariates. In such models, statistical significance of a particular covariate on the outcome variable is evaluated after effects of other covariates are already taken into account. In the present study, owing to the skewness of the outcome measures, their log-transformed scales were used as the dependent variables in the regression analysis to better satisfy the normal distribution assumption. The covariates considered in multiple linear regression models were age, race, gender, obesity status, waist-to-hip ratio (WHR), systolic blood pressure, diastolic blood pressure, total cholesterol, HDL cholesterol, LDL cholesterol, glucose, plasma ferritin, and renal function measures such as serum creatinine and UMACR. Stepwise variable-selection procedures were used to select covariates with the most important effect on the outcomes. A subgroup analysis was performed in the female participants to assess the effect of menopause on the outcome by fitting a multiple regression model with menopause (yes/no) in addition to all aforementioned covariates. For all the statistical analysis, a *P* value of 5% or less was considered statistically significant. SAS 9.2 for Windows was used to analyze the data.

Results

Urinary catalytic iron was significantly higher among the obese individuals compared to the nonobese individuals. In the obese group, the mean UBDI/Cr was 463 nmol/mg (95% CI, 343–582 nmol/mg) [52.3 nmol/μmol (95% CI, 38.8–65.8 nmol/μmol)], which was significantly greater than the mean UBDI/Cr in the control groups, which was 197 nmol/mg (95% CI, 141–253 nmol/mg) [22.3 nmol/μmol (95% CI, 15.9–28.6 nmol/μmol)] $P < 0.001$. Among the obese individuals, 51% had UBDI/Cr >253 nmol/mg (28.6 nmol/μmol), the upper limit of the 95% CI. Among these patients the mean (SD) UMACR was 8.47 (8.43) μg/mg. Summaries of other characteristics of the 2 groups are listed in Table 1.

Pearson correlation coefficients were computed to detect possible associations between urinary catalytic iron (both on its original scale and log-transformed scale) and individual numerical measures. In particu-

Table 2. Detailed information on the parameter estimates from the linear regression model.

	Parameter estimates			
Variable	Parameter estimate	SE	*t*	Pr[a] > |*t*|
Intercept	−0.36	1.78	−0.20	0.84
Obesity status	1.22	0.38	3.24	0.001
WHR	4.64	2.13	2.17	0.03

[a] Pr, probability.

lar, no correlation was observed between the UMACR and excretion of catalytic iron in the urine ($r = -0.02$, $P = 0.74$). These associations were reevaluated in a full linear regression analysis in which all the covariates were present in the model simultaneously. This model was significant, with an overall $P = 0.01$. No significant difference was observed in UBDI/Cr between the obese and the control groups, whether age was categorized into quartiles or actual age was used. A stepwise model selection was performed using the inclusion/exclusion criterion of $\alpha = 5\%$. This overall reduced stepwise selection model was significant, with $P < 0.0001$. Two variables, obesity status and WHR, were significant (Table 2) with $P = 0.001$ and $P = 0.03$, respectively. Comparison of the UBDI/Cr between the postmenopausal (n = 44) and premenopausal (n = 101) women revealed no significant difference [331 (486) nm/mg vs 436 (656) nm/mg; $P = 0.35$].

Plasma ferritin concentrations were available in a subset of the patients. Ferritin measurements were not normally distributed in this population. The mean plasma ferritin in the control group did not differ significantly from that in the obese group (64.6 vs 85.6 μg/L, respectively; $P = 0.12$). No relationship was observed between plasma ferritin and UBDI/Cr ($r = -0.0838$; $P = 0.2469$).

Among the obese individuals, 52 patients had metabolic syndrome and 48 patients did not, according to the definition of the National Cholesterol Education Program Adult Treatment Panel III report *(24)*. In post hoc analysis, in individuals with metabolic syndrome UBDI/Cr and UMACR were not found to be significantly different from values seen in individuals without metabolic syndrome (Table 3). No significant difference in plasma ferritin concentrations was seen between study participants with the metabolic syndrome and those without the metabolic syndrome [80.3 (98.4) vs 80.6 (69.9) μg/L; $P = 0.9$]. The mean UBDI/Cr was slightly higher in obese black individuals (492 nmol/mg; 95% CI, 287–697 nmol/mg), [55.6 nmol/μmole (95% CI, 32.4–78.8 nmol/μmol] compared to obese white individuals (458 nmol/mg; 95% CI, 305–611 nmol/mg) [51.7 nmol/μmol (95% CI, 34.5–68.9 nmol/μmol)], and in obese women (mean, 507 nmol/mg; 95% CI, 357–657 nmol/mg) [57.3 nmol/μmol of urine creatinine (95% CI, 40.3–74.2 nmol/μmol)] compared to obese men (315 nmol/mg; 95% CI, 192–466 nmol/mg) [37.2 nmol/μmol; (95% CI, 21.7–52.7 nmol/μmol)]. However, these differences were not statistically significant. As shown in Table 3, no significant difference was observed in the UBDI/Cr between patients with UMACR >30 in the control and the obese groups (mean, 333.4 vs 203.2 nmol/mg, respectively).

Table 3. Urinary catalytic iron in various groups.[a]

	UBDI/Cr, nm/mg	*P*
Blacks, obese group (n = 41)	492 (651)	0.78
Whites, obese group (n = 57)	476 (576)	
Blacks, control group (n = 17)	184 (292)	0.83
Whites, control group (n = 81)	200 (283)	
With the metabolic syndrome (n = 52)	543 (623)	0.2[b]
Without the metabolic syndrome (n = 48)	389 (579)	
Premenopausal women (n = 101)	331 (486)	0.35
Postmenopausal women (n = 44)	436 (656)	
Males, obese group (n = 23)	315 (310)	0.06
Females, obese group (n = 77)	507 (661)	
Males, control group (n = 31)	191 (251)	0.88
Females, control group (n = 68)	200 (297)	

[a] Values are presented as mean (SD).
[b] Among the obese participants only.

Discussion

The results of this study demonstrate that urinary catalytic iron is increased in obese individuals compared with nonobese individuals. This increase is independent of age and hypertension. As expected, significant mean differences were observed between the control and obese groups for variables such as glucose, systolic and diastolic blood pressure, total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides (Table 1). After we adjusted for covariates by using multiple regression analysis, the only factors that remained associated with increased UBDI/Cr were obesity and WHR. Individuals in the obese group in our study had considerably higher HDL cholesterol concentrations than expected. Twelve of the 100 obese individuals were on lipid-lowering agents. Because microalbuminuria is used as an early biomarker of kidney disease and CVD, and urine microalbumin concentrations are

above the reference interval in some obese individuals, we measured urine microalbumin in the study participants. We found no correlation between UBDI/UCr and UMACR results in our study, possibly owing to the variations in the values of both UMACR and UBDI/UCr. Limitations of a small sample size also may be part of the explanation for this lack of correlation.

Iron has been implicated recently in a variety of disease states. For example, high body-iron stores also have been linked to insulin resistance *(25, 26)*, metabolic syndrome *(25, 27–29)*, and diabetes *(18)*. However, it should be emphasized that epidemiological data have provided conflicting results, with results of several reported studies indicating an association between iron stores and progression of carotid atherosclerosis or acute myocardial infarction *(30, 31)*, whereas other study results have provided evidence against such an association *(32, 33)*. Perhaps an explanation is that iron status per se does not reflect the susceptibility to disease but rather it is the iron available to catalyze free-radical reactions that is important *(19)*. Similarly, iron has been associated with CVD *(15, 34)*. Our results did not show any difference in urinary catalytic iron between obese individuals with and those without metabolic syndrome.

On the basis of results of animal studies and limited preliminary human studies, catalytic iron has been causatively linked to kidney disease *(11, 12)* and CVD *(13–15)*. We therefore believe that it is of some interest to report the observation that catalytic iron is increased in obese individuals. There was a small but statistically significant difference in mean age between our obese and control groups. However, we found no significant difference in UBDI/Cr when compared across age quartiles between the obese and the control groups. Similarly we found no significant difference in UBDI/Cr between the postmenopausal and premenopausal women. These are limited, preliminary observations and there is insufficient information to date to enable us to understand the importance of the relationship of this observation to microalbuminuria.

Similarly, although an increase in plasma ferritin has been described in patients with metabolic syndrome *(29, 35)*, the high ferritin is more likely to be a reflection of an acute-phase reactant (related to the chronic inflammatory state) rather than iron stores *(36)*. We have measured the active form of ferric iron because this is what plays a pathological role in DM and its complications, rather than simply reflecting body stores. Serum ferritin concentrations have been reported to be higher in postmenopausal women compared to premenopausal women, in part due to cessation of menses *(37)*.

Previous studies have shown high urinary catalytic iron in DM *(38)*, CKD *(38)*, and CVD *(13)*. The source of increased iron in the kidney is not yet well understood. Urinary transferrin has been suggested as a source by Alfrey et al. *(39)*. Glomerular permeability-selectivity impairment due to injury leads to the movement of proteins such as transferrin into the urinary space. At a pH below 6.5, iron has been shown to be dissociated from transferrin. While urine courses along the nephron, and as the pH decreases, iron is released from transferrin *(39, 40)*, providing a source of iron that could act on renal tubular epithelial cells or be reabsorbed into the tubules. In an in vivo study, administration of an iron chelator, deferoxamine, prevented the increase in BDI in glomeruli and provided protection against proteinuria in a model of puromycin–aminonucleoside-induced minimal change nephrotic syndrome *(21)*. Administration of an oral iron chelator, Deferiprone *(17)*, effectively suppressed active experimental autoimmune encephalitis.

The BDI assay offers several advantages. The binding of the bleomycin-iron complex to DNA makes the reaction site specific, and interference from antioxidants is rare. Pertinent in this context are the reports *(41)* that there is no interference from various iron-containing proteins, including transferrin, ferritin, and hemoglobin. Application of the bleomycin assay shows that blood serum and plasma prepared from healthy individuals contains minimal amounts of BDI *(9, 13, 41, 42)*, and only modest amounts are found in the urine *(12, 41)*. Iron-containing proteins such as ferritin, myoglobin, hemoglobin, and cytochrome P-450 do not register in the assay, and thus the assay measures only iron capable of catalyzing free-radical reactions *(9, 19–21, 42)*. Thus the BDI assay offers a major advantage over measuring ferritin, which gives no indication of how much iron is available to participate in free-radical reactions, and may also be altered in response to inflammation.

Our study results demonstrate that high urinary catalytic iron in obesity provides a potential underlying biochemical explanation for the known clinical associations of obesity with cardiovascular and kidney diseases, independent of DM. Our data are consistent also with previous data suggesting that oxidative stress may be one of the underlying factors in the development of comorbidities of obesity *(28, 38)*, and catalytic iron may be a marker of oxidative stress.

LIMITATIONS

One of the limitations of our results is that they are derived from a cross-sectional study. We do not have data on plasma iron or hemoglobin concentrations among our study participants. We measured plasma ferritin in our patients and found a mild increase in the obese groups, with the data being skewed because some individuals had much higher concentrations of ferritin,

which is compatible with data in the literature *(43)*. However, we found no correlation between UBDI/Cr and plasma ferritin. The latter is well known to be an acute-phase reactant and may reflect acute or chronic inflammation. Our animal studies have indicated that catalytic iron concentrations in tissues are not related to iron stores *(19)*. There was very minimal use of over-the-counter or prescription forms of iron, vitamins, and antioxidants among the participants in our study. On the other hand there are no data to suggest that consumption of such substances is likely to be different in obese than nonobese individuals.

In summary, we have demonstrated that urinary catalytic iron is increased in obese individuals compared to nonobese individuals independently of other comorbidities and plasma ferritin. Because this abnormality is common-path physiologically linked to oxidative stress and could be a hallmark of tissue injury, additional research is required to determine if these changes are associated with the long-term complications of obesity.

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