Demonstration of a Hyperglycemia-Driven Pathogenic Abnormality of Copper Homeostasis in Diabetes and Its Reversibility by Selective Chelation

Quantitative Comparisons Between the Biology of Copper and Eight Other Nutritionally Essential Elements in Normal and Diabetic Individuals

Garth J.S. Cooper,[1,2,3,4] Yih-Kai Chan,[1,4] Ajith M. Dissanayake,[5] Fiona E. Leahy,[1,4,6] Geraldine F. Keogh,[1,4] Chris M. Frampton,[7] Gregory D. Gamble,[3] Dianne H. Brunton,[1] John R. Baker,[5] and Sally D. Poppitt[3,4]

We recently showed that treatment with the Cu^{II}-selective chelator, trientine, alleviates heart failure in diabetic rats, improves left ventricular hypertrophy in humans with type 2 diabetes, and increases urinary Cu excretion in both diabetic rats and humans compared with nondiabetic control subjects. In this study, we characterized the homeostasis of Cu and eight other nutritionally essential elements in diabetes under fully residential conditions in male subjects with type 2 diabetes and age-matched control subjects. We then probed elemental balance with oral trientine in a parallel-group, placebo-controlled study in these subjects. Before treatment, there were no detectable between-group differences in the balance of any element, although urinary output of several elements was greater in diabetic subjects. Mean extracellular superoxide dismutase (EC-SOD) activity was elevated in diabetic subjects, and its activity correlated strongly with the interaction between $[Cu]_{serum}$ and HbA_{1c}. Trientine caused the Cu balance to become negative in diabetic subjects through elevated urinary Cu losses and suppressed elevated EC-SOD. Basal urinary Cu predicted urinary Cu losses during treatment, which caused extraction of systemic Cu^{II}. We suggest that cardiovascular complications in diabetes might be better controlled by therapeutic strategies that focus on lowering plasma glucose and loosely bound systemic Cu^{II}. *Diabetes* 54: 1468–1476, 2005

Oxidative stress has been implicated as a contributory mechanism in age-related disorders, including diabetes, hypertension, obesity, and atherosclerosis (1). Clinical trials of antioxidant (2) or carbonyl-trapping (3) agents in these disorders have had mixed success, indicating that the mechanisms underlying these disorders may be more complex than previously thought (3). In diabetes, the nature of oxidant stress and how it might promote complications is still unclear.

Free Fe and Cu ions are highly redox active (4) and might contribute to tissue damage by the generation of reactive oxygen species (ROS) (1), but the in vivo availability of catalytic Fe and Cu is usually very restricted (4). Both metals have been previously discussed in relation to the mechanisms of diabetes complications (5), but abnormalities of Fe homeostasis have not been linked to the major classes of diabetes and it is unknown whether altered Cu metabolism is relevant to diabetes complications.

Heart disease leads to death in most diabetic subjects (6,7). The Cu-selective chelator, trientine, causes a Cu^{II}-trientine complex to appear in the urine of diabetic rats whose abnormal Cu metabolism occurred after diabetes induction (8); trientine alleviated heart failure, improved cardiomyocyte structure, and reversed elevations in left ventricle collagen and β_1 integrin without lowering blood glucose (8). Trientine also increased Cu excretion and

From the [1]School of Biological Sciences, Faculty of Science, University of Auckland, New Zealand; the [2]Centre for Molecular Biodiscovery, Faculty of Science, University of Auckland, New Zealand; the [3]Department of Medicine, Faculty of Medical and Health Sciences, University of Auckland, New Zealand; the [4]Human Nutrition Unit, University of Auckland, New Zealand; the [5]South Auckland Diabetes Service, Middlemore Hospital, Otahuhu, New Zealand; the [6]School of Medical Sciences, Faculty of Medical and Health Sciences, University of Auckland, New Zealand; and the [7]Department of Medicine, Christchurch Clinical School, University of Otago, Christchurch, New Zealand.

Address correspondence and reprint requests to Garth J.S. Cooper, Level 4, Thomas Bldg., School of Biological Sciences, University of Auckland, Private Bag 92019 Auckland, New Zealand. E-mail: g.cooper@auckland.ac.nz.

Received for publication 9 December 2004 and accepted in revised form 2 February 2005.

G.J.S.C. is a stock shareholder in, has served on an advisory panel or board of, and has received grant research support from Protemix. Y.-K.C. is employed by and has received grant/research support from Protemix. G.D.G. has received fees for providing statistical support to Protemix. J.R.B. is employed by, has served on the advisory panel or board of, and is a stock shareholder in Protemix. D.H.B. has been paid a consulting fee for statistical analysis by Protemix. S.D.P. has received grant/research support from Protemix. C.M.F. is associated with Protemix. Protemix is not affiliated with Anstead U.K., Ltd.

EC-SOD, extracellular superoxide dismutase; FPG, fasting plasma glucose; IBC, iron-binding capacity; PABA, *p*-aminobenzoic acid; REML, restricted maximum likelihood; RIGLS, restrictive iterative generalized least squares; ROS, reactive oxygen species.

TABLE 1
Pretreatment (days 1 and 7) age, BMI, serum analyte concentrations, and hematologic indexes in control and diabetic subjects

	Control	Diabetic	*P*
Age (years)	46.9 ± 10.6	50.7 ± 10.8	NS
Diabetes duration (years)	N/A	6.7 (1–34)	—
BMI (kg/m^2)	26.2 ± 4.9	31.3 ± 4.3	<0.001
Fasting plasma glucose (mmol/l)	4.8 ± 0.5	10.1 ± 3.8	<0.001
HbA_{1c} (%)	5.3 ± 0.27	9.2 ± 2.2	<0.001
EC-SOD (units/l)	32.2 ± 26.9	85.2 ± 115	<0.01
Albumin (g/l)	41.6 ± 2.0	41.3 ± 2.1	NS
IBC (μmol/l)	54.7 ± 8.1	57.2 ± 7.3	NS
Ferritin (μg/l)	127 ± 94.8	388 ± 271	<0.001
Cu (μmol/l)	15.5 ± 2.8	16.0 ± 2.4	NS
Fe (μmol/l)	18.0 ± 4.5	17.3 ± 4.9	NS
Zn (μmol/l)	12.1 ± 2.1	12.5 ± 2.2	NS
Ca (mmol/l)	2.22 ± 0.15	2.30 ± 0.27	NS
Mg (mmol/l)	0.81 ± 0.06	0.75 ± 0.09	<0.01
Mn (μmol/l)	0.01 ± 0.02	0.01 ± 0.01	NS
Se (μmol/l)	1.11 ± 0.20	1.20 ± 0.25	NS
Hb (g/l)	152 ± 9.7	150 ± 13.2	NS
Packed cell volume (%)	45.2 ± 2.9	44.1 ± 3.9	NS
Mean corpuscular volume (fl)	90.5 ± 3.8	85.3 ± 4.3	<0.001
White blood cells (× 10^9/l)	6.8 ± 2.7	6.3 ± 1.3	NS
Platelets (× 10^9/l)	248 ± 50	234 ± 55	NS

Data are means ± SD or mean (range). There were 20 subjects each in the diabetic and control groups. Concentrations of Cu, Zn, Ca, Mg, Mn, and Se were determined by inductively coupled plasma–mass spectrometry. $[Mn]_{serum}$ was near the minimum detectable concentration. $[Mo]_{serum}$ and $[Cr]_{serum}$ are not included, as their values were at or below the respective minimum detectable concentration values. *P* values were determined with a one-way ANOVA (two-tailed tests). N/A, not applicable.

decreased left ventricle mass in diabetic humans (8), implicating increased systemic Cu^{II} in the mechanism by which diabetes damages the heart.

Here we detail measurements of a 6-day balance of nine elements, including Cu and Fe, in diabetic and age-matched nondiabetic control subjects in whom we probed systemic metal balance with oral trientine in a subsequent study. Basal urinary output of Cu and Fe was significantly increased in diabetes, and those output values correlated strongly. Trientine treatment increased urinary excretion of Cu in a dosage-dependent manner, as predicted by basal urinary Cu, thereby causing a positive Cu balance to become negative in diabetes. In contrast, it modified neither Fe balance nor rates of urinary or fecal Fe excretion in these subjects. Furthermore, trientine did not render negative the balance of any other element in either diabetic or control subjects. Regulation of Cu metabolism was shown to be abnormal in diabetes and selectively modified by trientine, which did not concomitantly modify Fe metabolism. These findings are consistent with the effects of trientine in reversing the tendency to systemic accumulation of increased, loosely bound Cu^{II} in diabetes, which may help explain its therapeutic effects in diabetic cardiovascular disease.

RESEARCH DESIGN AND METHODS

Men (age 30–70 years) with normal electrocardiograms were recruited for this study. Diabetic subjects had been diagnosed at least 6 months prior to the study, and control subjects had normal glucose tolerance. Exclusion criteria included the presence of type 1 diabetes; nephropathy; abnormal hematology or Fe deficiency; a history of significant cardiac disease; previous hepatic, gastrointestinal, or endocrine disease other than diabetes; gangrene or active sepsis; severe retinopathy; nondiabetic renal disease or renal allograft; malignancy, except cutaneous basal cell carcinoma; or known abnormality of Cu or Fe metabolism as well as current treatment with diuretics or calcium-channel blockers.

All protocols received appropriate regulatory approval, and all subjects provided written informed consent.

Elemental balance studies. Type 2 diabetic (n = 20) and control (n = 20) subjects underwent a factorial, randomized, double-blind, placebo-controlled elemental balance study with screening, enrollment, run-in, treatment, and follow-up periods. Participants were resident throughout the study. Elemental intake was controlled by providing all items of food and beverages, which were directly measured (duplicate diets). Diets (Xyris Foodworks) were constructed to adhere to American Diabetes Association recommendations (9).

Elemental excretion was determined throughout the basal period. Balances were calculated from the difference between intake and output. $[Elements]_{fasting\ serum}$ were determined on the mornings of days 1 and 7, the latter before drugs were administered. After the basal study (days 1–6), subjects were randomized to placebo or trientine (2,400 mg/day; Anstead, Essex, U.K.) and immediately entered the 6-day treatment period (days 7–12). The second part of the study was an otherwise identical regimen, which was completed on the morning of day 13.

Sample acquisition and analysis. Completeness of urine collections was confirmed by a *p*-aminobenzoic acid (PABA) test. Feces were freeze-dried; completeness was considered to be 93–97% (opaque food-markers; X-ray of fecal samples). Elemental concentrations in urine, feces, and food aliquots were measured (10) using an inductively coupled plasma–mass spectrometer (Perkin-Elmer Sciex Elan 6100). Biochemical and hematological variables (Table 1) were measured in fasting blood samples on days 1 and 7 (pretreatment) and day 13 (treatment). The level of 2-h $[Mg]_{serum}$ was determined for 10-h after dosing. Data reported for day 1 are herein termed "basal" and those for days 1 and 7 are designated "pretreatment."

Dosage-dependent urinary metal excretion. We also measured the effects of increasing dosages of trientine (300, 600, 1,200, and 2,400 mg/day) on urinary elements. Samples were collected before and after each 1-week treatment period from control and diabetic subjects (n = 7 each) after completion of the main study (total duration 28 weeks).

Statistical analysis. Basal values from healthy (control) and diabetic patients were compared by one-way ANOVA. Restrictive iterative generalized least squares (RIGLS) models were fitted by restricted maximum likelihood (REML) (11). The effects of patient status (control or diabetic) and treatment (placebo or trientine) were analyzed using a factorial experimental design with two time periods, days 0–6 and 7–12, wherein the interaction effect was the term of primary significance. Differences between time periods for each subject were used in a mixed-model ANOVA fitted by REML in which subjects were considered as random (12) and differences between treatments (trien-

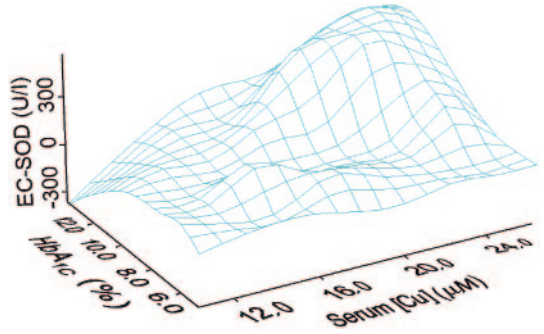

FIG. 1. Spline fine-grid response surface fitted to a three-dimensional plot (S-Plus, version 6.1) of the relation between $[Cu]_{serum}$ (O_x), HbA_{1c} (O_y), and serum EC-SOD activity (O_z) in diabetic subjects ($n = 20$) on day 7.

tine or placebo) and patient status (diabetic or control) and the interaction between treatment and patient status were tested. Assumptions of normality and homoscedasticity were directly verified. Pearson's correlation coefficients were calculated to determine the relation between basal Cu and key variables. A model predicting urinary Cu excretion during drug treatment (days 7–12) was constructed by adding predictive basal (day 1) variables into a forward, stepwise, multiple regression model (with $\alpha = 0.25$ for entry and $\alpha = 0.10$ for leaving). A significance level of $\alpha = 0.05$ was used for all statistical tests.

RESULTS

Basal group characteristics. In all, 20 diabetic and 19 control subjects completed the study. Diabetic subjects had greater BMI, fasting plasma glucose (FPG), and HbA_{1c} than did control subjects (each $P < 0.001$) (Table 1). Basal $[Mg]_{serum}$ was lower ($P < 0.01$) and $[ferritin]_{serum}$ was elevated ($P < 0.001$) in diabetic compared with control subjects. Serum levels are usually not a good measure of nutritional status, and measured values of Cu and five other elements did not differ between diabetic and control subjects (Table 1).

Relation between serum HbA_{1c} and the interaction between extracellular superoxide dismutase and $[Cu]_{serum}$. Basal extracellular superoxide dismutase (EC-SOD) was elevated in diabetic subjects on days 1 and 7 (both $P < 0.01$) and was related to $[Cu]_{serum}$ levels ($EC\text{-}SOD = 19.6.[Cu]_{serum} - 228$; $r^2 = 0.16$, $P = 0.011$), but no equivalent association was observed in control subjects. Basal EC-SOD did not correlate with the serum concentration of any other element in diabetic subjects. EC-SOD was related to the interaction between $[Cu]_{serum}$ and HbA_{1c} in RIGLS models on days 1 ($[Cu]_{serum}$, $P = 0.022$; HbA_{1c}, $P = 0.014$; interaction term, $P = 0.0096$) and 7 ($[Cu]_{serum}$, HbA_{1c}, and interaction term, all $P < 0.0001$). A three-dimensional spline-surface (on day 7) illustrated that maximum elevations in EC-SOD occurred mainly in subjects in whom both $[Cu]_{serum}$ and HbA_{1c} were high (Fig. 1). Thus, elevated EC-SOD may reflect a chemical interaction between these variables. In diabetic subjects, trientine treatment lowered mean serum EC-SOD below baseline levels (31.8 ± 34.7 [day 13] vs. 72.6 ± 72.0 [day 1] units/l; $P = 0.034$). EC-SOD was not related to interactions between serum concentrations of any other elements and HbA_{1c} in diabetic subjects (RIGLS; all NS) nor was HbA_{1c} alone significantly related to EC-SOD in either diabetic or control subjects.

Serum ferritin. $[Ferritin]_{serum}$ was elevated in diabetic compared with control subjects (Table 1), but values were uncorrelated with $[Fe]_{serum}$, iron-binding capacity (IBC), $[Hb]_{blood}$, or packed cell volume in diabetic subjects. These findings are consistent with those in previous reports.

Basal elemental balance. Basal elemental balances are presented in Table 2. Food intake did not differ between groups or as a result of treatment, nor did the basal balance differ between control and diabetic groups. The Cu balance was variable but tended to be more positive in diabetic than in control subjects, although the difference was not significant ($P = 0.19$) (Table 2). Basal urinary excretion of Cu, Fe, Zn, Ca, Mn, Se, and Cr was higher in diabetic subjects, and basal urinary Cu excretion was closely correlated with that of Fe ($[Fe]_{24\ h\ urine} = 1.94.[Cu]_{24\ h\ urine} + 0.53$; $r^2 = 0.48$, $P = 0.00067$). Thus, increased basal urinary Cu excretion in diabetes is closely related to increased urinary Fe excretion. Diabetic subjects had elevated urinary volumes, but statistical modeling indicated that the increased urinary output was not a factor in the increased urinary elemental outputs.

Effects of drug treatment on elemental balance. The effects of drug treatment (placebo or trientine) and of interactions between trientine and metabolic status (control or diabetes) were examined by ANOVA (Table 3); Table 4 shows the effects of treatment on differences in balance and urinary and fecal excretion of Cu, Fe, Zn, and Ca.

Copper. Trientine did not modify the Cu balance in the whole study group, but the interaction term was significant ($P = 0.0028$) (Table 3). Trientine decreased the Cu balance in diabetic subjects compared with placebo ($P = 0.021$) (Table 4), but its effect on balance in control subjects was of marginal significance ($P = 0.065$) (Table 4). Basal urinary Cu was elevated in diabetic subjects ($P = 0.0011$) (Table 2). Trientine treatment modified urinary Cu excretion in all subjects ($P < 0.0001$), but the interaction was borderline ($P = 0.075$) (Table 3). Trientine stimulated urinary Cu excretion in both groups ($P < 0.0001$) (Table 4), indicating extraction of Cu^{II} in both. Moreover, its interaction with fecal Cu was significant ($P = 0.0034$) (Table 3). Fecal Cu effects were evoked largely via decreased control values ($P = 0.0041$) (Table 4). These observations are consistent with lower fractional absorption of Cu in diabetes.

Iron. Trientine modified the Fe balance ($P = 0.028$), but the corresponding trientine–metabolic status interaction term was not significant (Table 3). Trientine increased the Fe balance in control ($P = 0.034$) but not in diabetic subjects (Table 4). Basal urinary Fe was elevated in diabetic subjects ($P = 0.0001$) (Table 2), but was unaffected by trientine, which had only borderline effects in diabetic subjects ($P = 0.051$) (Table 4). Trientine modified fecal Fe in the whole group ($P = 0.019$) (Table 3), but the trientine–metabolic status interaction was insignificant. Trientine lowered fecal Fe excretion in control subjects ($P = 0.023$) (Table 4). These data suggest lower fractional absorption of Fe in diabetes.

Zinc. Trientine elicited significant modification of the Zn balance in all subjects ($P = 0.0021$), and the trientine–metabolic status interaction was significant ($P = 0.002$) (Table 3). Basal urinary Zn was elevated in diabetic subjects ($P < 0.0001$) (Table 2). Trientine modified urinary Zn in all subjects ($P < 0.0001$) (Table 3) by stimulation in

TABLE 2
Pretreatment (days 1–6) balance of nine elements with corresponding urinary and fecal outputs in diabetic and matched control subjects

	Control	Diabetic	*P*
Cu (μmol/6 days)			
Balance	1.49 (−25.0 to 28.0)	23.7 (0.9–46.4)	0.19
Urinary	1.16 (1.04–1.28)	1.59 (1.36–1.82)	0.0011
Fecal	168 (143–193)	159 (139–180)	NS
Fe (μmol/6 days)			
Balance	480 (180–779.4)	479.2 (189.4–769.1)	NS
Urinary	2.13 (1.81–2.46)	3.61 (2.98–4.25)	0.0001
Fecal	1,757 (1,481–2,033)	1,888 (1,624–2,153)	NS
Zn (μmol/6 days)			
Balance	−167.2 (−383.8 to 49.4)	−71.8 (−232.8 to 89.2)	NS
Urinary	50.4 (41.2–59.5)	125.7 (106.4–145.1)	<0.0001
Fecal	1,420 (1,212–1,628)	1,301 (1,134–1,469)	NS
Mn (μmol/6 days)			
Balance	−45.7 (−188 to 97.0)	90.0 (−35.7 to 218)	NS
Urinary	0.03 (0.0–0.05)	0.14 (0.07–0.21)	0.0016*
Fecal	958 (810–1,106)	889 (770–1,007)	NS
Mo (μmol/6 days)			
Balance	1.03 (−0.84 to 2.89)	2.87 (1.65–4.08)	0.092
Urinary	3.89 (3.05–4.72)	3.51 (2.70–4.33)	NS
Fecal	6.40 (5.22–7.57)	5.41 (4.32–6.50)	NS
Ca (mmol/6 days)			
Balance	15.2 (−7.4 to 46.3)	39.2 (14.4–64.0)	NS
Urinary	21.8 (18.5–25.2)	29.3 (22.8–35.8)	0.038
Fecal	159.5 (135.4–183.7)	141.9 (119.8–164.1)	NS
Mg (mmol/6 days)			
Balance	−0.28 (−13.3 to 12.7)	10.8 (−3.3 to 24.9)	NS
Urinary	31.0 (26.7–35.3)	29.0 (25.3–32.6)	NS
Fecal	83.1 (71.5–94.8)	92.3 (76.7–101.6)	NS
Se (μmol/6 days)			
Balance	−0.66 (−1.48 to 0.15)	−0.84 (−1.82 to 0.15)	NS
Urinary	2.07 (1.69–2.45)	2.74 (2.32–3.16)	0.018
Fecal	3.30 (2.66–3.94)	2.19 (1.79–2.59)	NS
Cr (μmol/6 days)			
Balance	2.26 (1.58–2.95)	2.61 (1.88–3.35)	NS
Urinary	0.00 (0.00–0.00)	0.012 (0.002–0.022)	0.018*
Fecal	2.82 (2.23–3.42)	2.90 (2.50–3.30)	NS

Data are means (95% CI). There were 20 subjects each in the diabetic and control groups. *P* was determined with a one-way ANOVA (two-tailed tests). *Urinary Mn and Cr were near their respective minimum detectable concentrations, so these results should be interpreted with caution.

both control ($P < 0.0001$) and diabetic ($P < 0.0001$) subjects (Table 4). Trientine modified fecal Zn excretion ($P < 0.0001$) (Table 3), and the trientine−metabolic status interaction was significant ($P = 0.0045$). Trientine decreased fecal Zn in control subjects ($P = 0.0001$) (Table 4). These data suggest that fractional Zn absorption is lower in diabetes.

Calcium. Trientine significantly modified the Ca balance ($P = 0.0022$) (Table 3), mainly by increases in control subjects ($P = 0.0012$) (Table 4). Drug−metabolic status interactions were significant for Ca balance ($P = 0.033$) and fecal excretion ($P = 0.020$) (Table 3). Trientine modified fecal Ca excretion ($P = 0.0014$) (Table 3), mainly via decreased control values ($P = 0.00065$) (Table 4). Fractional Ca absorption may thus be lower in diabetes.

Magnesium. The basal Mg balance did not differ between diabetic and control subjects, nor was it modified by trientine treatment. Indexes of Mg balance were uncorrelated with HbA_{1c} or FPG in either group (data not shown).

Manganese. Trientine modified the Mn balance ($P = 0.028$) and fecal Mn excretion ($P = 0.017$) (Table 3). The trientine−metabolic status interaction was significant for Mn balance ($P = 0.043$) and fecal excretion ($P = 0.034$) (Table 3). Trientine altered the Mn balance mainly via decreased fecal excretion. The effects on Mn and Ca balance were similar, being evoked mainly via decreased fecal excretion in control subjects and consistent with decreased fractional gut absorption of Mn in diabetic subjects.

Selenium. The trientine−metabolic status interaction was significant for Se balance ($P = 0.016$) and fecal excretion ($P = 0.013$) (Table 3). The effects on Se balance resembled those for Ca and Mn, being evoked mainly via decreased fecal Se excretion in control subjects. Trientine did not modify $[Se]_{serum}$ (data not shown).

Molybdenum/chromium. Neither diabetes nor trientine altered the Mo or Cr balance or their urinary or fecal excretion rates (data not shown). Neither FPG nor HbA_{1c} correlated with indexes of Cr balance in either diabetic or control subjects (data not shown).

Dosage-dependent effects of trientine on metal excretion. Trientine increased urinary Cu in both diabetic

TABLE 3
Effects of trientine treatment on differences in balance and in urinary and fecal excretion of Cu, Fe, Zn, Mn, Ca, and Se in control and diabetic subjects

	Overall model		Factor tests					
			Control/diabetes		Placebo/trientine		Interaction	
	$F_{3,36}$	P	$F_{1,36}$	P	$F_{1,36}$	P	$F_{1,36}$	P
Cu								
Balance	3.61	0.022	0.04	NS	0.50	NS	10.28	0.0028
Urinary excretion	37.74	<0.0001	3.49	0.07	106.36	<0.0001	3.37	0.075
Fecal excretion	4.05	0.014	0.24	NS	2.09	NS	9.82	0.0034
Fe								
Balance	2.02	NS	0.09	NS	5.25	0.028	0.71	NS
Urinary excretion	1.92	NS	0.23	NS	2.09	NS	3.43	0.072
Fecal excretion	2.34	0.090	0.04	NS	6.06	0.019	0.91	NS
Zn								
Balance	7.37	0.0006	0.00	NS	11.01	0.0021	11.10	0.002
Urinary excretion	27.14	<0.0001	4.14	0.049	73.62	<0.0001	3.66	0.064
Fecal excretion	11.76	<0.0002	0.07	NS	26.02	<0.0001	9.19	0.0045
Mn								
Balance	3.24	0.033	0.10	NS	5.22	0.028	4.40	0.043
Urinary excretion	0.84	NS	2.30	NS	0.00	NS	0.23	NS
Fecal excretion	3.75	0.019	0.17	NS	6.24	0.017	4.85	0.034
Ca								
Balance	5.25	0.0042	0.00	NS	10.84	0.0022	4.90	0.033
Urinary excretion	0.60	NS	0.82	NS	0.32	NS	0.67	NS
Fecal excretion	5.97	0.0021	0.08	NS	11.96	0.0014	5.88	0.020
Se								
Balance	3.99	0.015	4.47	0.041	1.06	NS	6.44	0.016
Urinary excretion	1.41	NS	2.77	NS	0.10	NS	1.35	NS
Fecal excretion	3.74	0.019	1.80	NS	2.51	NS	6.91	0.013

Values determined with two-way ANOVA. Models were fitted by restricted maximum likelihood. No significant effects on Mo, Mg, or Cr were present, so their values have not been included. In the overall model, we excluded from analysis the one control subject who did not complete the treatment aspect of the study.

($[Cu]_{24\ h\ urine}$ = 0.00245.[trientine dosage] + 0.192; r^2 = 0.66, $P < 0.0001$) and nondiabetic ($[Cu]_{24\ h\ urine}$ = 0.00183.[trientine dosage] + 0.177; r^2 = 0.56, $P < 0.0001$) subjects in a dosage-dependent manner; gradients for Cu excretion did not differ between groups. Trientine stimulated urinary Zn in both diabetic ($[Zn]_{24\ h\ urine}$ = 0.016.[trientine dosage] + 14.5; r^2 = 0.54, $P < 0.0001$) and nondiabetic ($[Zn]_{24\ h\ urine}$ = 0.0097.[trientine dosage] + 4.32; r^2 = 0.42, $P < 0.0001$) groups in a dosage-dependent manner; the diabetic gradient was significantly greater ($P < 0.05$). These findings are consistent with those previously reported (13). Trientine had no dosage-dependent effect on 24-h urinary excretion of Fe, Mn, Ca, Mg, Mo, Se, or Cr (data not shown), nor did it modify $[Mg]_{serum}$ over 10 h (data not shown).

Basal variables predicting cupriuretic response to trientine. We calculated a multivariate regression model relating basal variables to urinary Cu excretion during drug treatment (Table 5). Trientine and diabetes were positive factors; urinary Cu excretion on days 1–6 and basal $[Mg]_{serum}$ were significant, positively continuous components and basal $[ferritin]_{serum}$ was negative. The mechanisms relating basal $[Mg]_{serum}$ and $[ferritin]_{serum}$ to urinary Cu are uncertain.

DISCUSSION

In this study, we found that several aspects of Cu metabolism were altered in diabetic subjects. Basal urinary Cu was 1.4-fold higher in diabetic than in control subjects, whereas $[Cu]_{serum}$ did not differ significantly between the two groups. These findings are consistent with some previous reports (14,15), but others have reported that diabetic subjects with complications have elevated $[Cu]_{serum}$ (16).

There was a trend for the Cu balance to be elevated in diabetic subjects, as well as a significant effect of interaction between trientine and diabetes on Cu balance. Trientine markedly stimulated urinary Cu in both subject groups, but lowered the Cu balance only in diabetic subjects. Urinary Cu excretion during drug treatment was positively correlated with pretreatment urinary Cu levels. Thus, elevated basal urinary Cu predicted drug-induced cupriuresis; individual responses may have been determined by prior systemic Cu^{II} accumulation. In contrast, trientine decreased fecal Cu in control subjects only, possibly through increased uptake, consistent with known actions of polyvalent chelators to increase metal absorption (17). An alternative mechanism that could contribute to the observed effects of trientine on fecal metal excretion is that diabetes may modify hepatobiliary excretion of some or all of the elements concerned, although we are unaware of data to support this hypothesis. Thus, regulation of Cu homeostasis differed significantly between groups, and trientine elicited effects to reverse the elevated Cu balance in diabetic subjects, mainly through stimulation of urinary Cu excretion.

Ceruloplasmin (ferro-O_2-oxidoreductase; EC 1.16.3.1) is a circulating Cu protein present in vertebrate plasma (18).

TABLE 4
Effects of placebo and trientine treatment on changes in balance and in urinary and fecal excretion of Cu, Fe, Zn, and Ca between pretreatment (days 1–6) and treatment (7–12) periods in control and diabetic subjects

	Control		Diabetic	
	Mean (95% CI)	*P*	Mean (95% CI)	*P*
Cu (μmol/6 days)				
Placebo				
ΔBalance	−35.8 (−60.7 to −10.9)	—	15.2 (−18.4 to 48.8)	—
ΔUrinary excretion	0.1 (−0.2 to 0.3)	—	0.2 (−0.4 to 0.7)	—
ΔFecal excretion	36.3 (12.2–60.3)	—	−15.0 (−44.6 to 14.6)	—
Trientine				
ΔBalance	1.47 (−33.5 to 36.5)	0.065	−43.3 (−83.1 to −3.5)	0.021
ΔUrinary excretion	23.0 (17.4–28.7)	<0.0001	33.1 (22.2–43.9)	<0.0001
ΔFecal excretion	−28.5 (−66.0 to 8.9)	0.0041	8.9 (−26.3 to 44.1)	NS
Fe (μmol/6 days)				
Placebo				
ΔBalance	−227 (−566 to 113)	—	−50.6 (−330 to 229)	—
ΔUrinary excretion	−0.02 (−0.51 to 0.47)	—	−0.33 (−0.67 to 0.01)	—
ΔFecal excretion	235 (−94.3 to 563.8)	—	51.9 (−206 to 310)	—
Trientine				
ΔBalance	257 (−79.8 to 593)	0.034	173 (−252 to 598)	NS
ΔUrinary excretion	−0.11 (−0.49 to 0.26)	NS	0.40 (−0.31 to 1.12)	0.051
ΔFecal excretion	−306 (−671 to 58.4)	0.023	−187 (−641 to 266)	NS
Zn (μmol/6 days)				
Placebo				
ΔBalance	−202 (−344 to −59.7)	—	100 (−108 to 308)	—
ΔUrinary excretion	4.5 (−1.3 to 10.4)	—	6.6 (−10.5 to 23.8)	—
ΔFecal excretion	202 (67.7–336)	—	−107 (−306 to 92.2)	—
Trientine				
ΔBalance	412 (177–648)	<0.0001	98.9 (−137 to 335)	NS
ΔUrinary excretion	121 (81.5–161)	<0.0001	190.4 (124–256)	<0.0001
ΔFecal excretion	−562 (−820 to −304)	0.0001	−302 (−539 to −63.7)	NS
Ca (mmol/6 days)				
Placebo				
ΔBalance	−27.2 (−47.9 to −6.4)	—	−2.7 (−24.1 to 18.7)	—
ΔUrinary excretion	−2.6 (−4.7 to −0.57)	—	−2.7 (−6.3 to 0.85)	—
ΔFecal excretion	30.5(11.9–49.1)	—	5.7 (−14.0 to 25.4)	—
Trientine				
ΔBalance	35.2 (4.8–65.6)	0.0012	9.5 (−19.0 to 38.0)	NS
ΔUrinary excretion	−2.3 (−5.6 to 1.1)	NS	−4.7 (−8.3 to −1.1)	NS
ΔFecal excretion	−37.4 (−69.7 to −5.1)	0.00065	−6.2 (−37.0 to 24.5)	NS

Data are means (95% CI); *n* as in Table 3. Determined with one-way ANOVA. Exact *P* values are given for trientine treatment vs. corresponding placebo treatment.

Normally, ≥95% of plasma Cu is ceruloplasmin bound, and levels of circulating Cu and ceruloplasmin are closely related (19). These are the biomarkers most frequently used as measures of Cu status, and both are depressed in severe Cu deficiency (20). However, levels plateau when Cu intake is adequate and do not reflect the magnitude of Cu intake beyond this point, so they are not useful for characterizing Cu excess states (21). In one study, neither biomarker decreased in humans fed a marginally low Cu diet for >3 months (22). Based on our study, we conclude that measurements of isolated $[Cu]_{serum}$ or $[ceruloplasmin]_{serum}$ values are unlikely to be informative concerning the presence of probable systemic Cu^{II} excess in diabetic subjects.

TABLE 5
Multivariate regression model predicting urinary Cu excretion during days 7–12 from basal (day 1) variables

	β coefficient	SE	*t*	*P*
Intercept	—	15.48	−2.88	0.007
Drug:placebo	0.82	2.20	12.6	<0.001
Diabetes:control	0.20	2.91	3.21	0.003
Urinary Cu excretion (days 1–6)	0.22	3.02	2.86	0.007
Basal $[Ferritin]_{serum}$	−0.32	0.01	−4.13	<0.001
Basal $[Mg]_{serum}$	0.20	0.02	2.65	0.012

Summary of the multivariate regression model is as follows: r^2 = 0.862; SE of the estimate = 6.78 (P = 0.001).

Although Cu is an essential trace nutrient, it is also a potent cytotoxin when excess amounts accumulate in tissues (23). Because of its redox chemistry, Cu^{II} readily participates in reactions that elicit ROS production (4,24). Possible roles for Cu-catalyzed redox reactions in diabetes complications have been discussed previously (3,5), and the relative importance of Cu in diabetes in vivo was shown when we demonstrated the reversal of heart failure by systemic Cu^{II} chelation in diabetic rats (8).

In our study, EC-SOD activity was elevated in diabetic subjects and correlated with an HbA_{1c}-$[Cu]_{serum}$ interaction. This finding is consistent with a mechanism whereby

elevated EC-SOD is related to a $[Cu]_{serum}$−chronic hyperglycemia association, with the latter factor being accepted as a driver of the tendency to develop diabetes complications (25). EC-SOD, a secretory glycoprotein, is the major SOD isoenzyme in extracellular fluids (26) and blood vessel walls (27). Activity and concentrations of serum EC-SOD are known to be elevated in diabetic subjects (26,28), and a significant association between the serum concentration of EC-SOD and the severity of vascular complications has been previously reported (29). A correlation between $[Cu]_{serum}$ and EC-SOD activity has been reported in humans with essential hypertension (30). Serum EC-SOD activity is reportedly correlated with diabetes duration, carotid artery intimal-media thickness, and severity of nephropathy and retinopathy. It has also been proposed as a marker of vascular injury, possibly reflecting hyperglycemia-induced oxidative injury to the vascular endothelium (29). Nitric oxide (NO), a key physiological vasodilator (31), reacts with superoxide anion at an extremely rapid rate (1,27). The balance between superoxide anion concentrations and cellular antioxidant capacity, particularly SOD activity, is likely to regulate the bioactivity of NO (31). As the major SOD isoform present in vascular endothelium, where it acts to regulate superoxide levels, EC-SOD is a key regulator of endothelium-derived NO bioactivity in blood vessels (27).

Elevated EC-SOD levels (26,28) reflect increased superoxide production in diabetes (32). The finding of decreased EC-SOD after trientine treatment is consistent with the suppression of vascular superoxide production, and our current study implicates an interaction between $[Cu]_{serum}$ and chronic hyperglycemia in the mechanism by which diabetes causes vascular damage. Trientine treatment could suppress intravascular consumption of NO by lowering vascular superoxide production, thereby enhancing physiological vasodilatation, which is defective in diabetes (32). Trientine treatment lowered EC-SOD activity to control values in diabetic subjects. These data, taken in conjunction with data from our previous study demonstrating that chronic trientine treatment reverses established heart failure in diabetic rats and left ventricular hypertrophy in diabetic humans and rats (8), support the therapeutic lowering of systemic Cu^{II} for suppression of vascular damage. Because disease mechanisms in hypertensive heart disease, ischemic cardiomyopathy, and atherosclerosis are similar to those in diabetic heart disease (1,7), we expect that our results could also prove relevant in these other related conditions (8).

Basal balances of nine essential elements (33–35) were equivalent between study groups, although the basal urinary excretion rates for Cu, Fe, Zn, Ca, Mn, Se, and Cr were significantly elevated in the diabetic group. Trientine treatment significantly increased the Zn balance in control subjects, mainly via decreased fecal excretion, whereas it stimulated urinary Zn output in both groups. Previous findings of hyperzincuria and lower Zn absorption in diabetic animals and humans have prompted conjecture that diabetic subjects might be more susceptible to Zn deficiency (36); however, others have reported increased tissue Zn values after trientine treatment (37). Apparent absorption and retention of Cu and Zn have been reported in diabetic rats in which food consumption was twice that of controls, and the fractional absorption of Zn and Cu was reported as being lower. In one study (37), net absorption was higher, although it was offset by higher urinary excretion so that the final Cu/Zn retention was similar in both groups. These findings are similar to our results. Low fractional absorption of Zn in diabetic rats has been attributed to lower intestinal transport associated with increased concentrations of intestinal metallothionein, an inhibitor of Cu and Zn transport (36). The increased metallothionein content in the enterocytes of diabetic patients is a plausible mechanism for the lesser degree of stimulation by trientine treatment of Cu and Zn uptake in diabetic subjects. This mechanism could protect against increased systemic uptake that might otherwise result from diabetic hyperphagia. We observed similar disparities in the effects of trientine on the uptake of Ca and Fe between control and diabetic subjects. Similar mechanisms may thus operate in diabetes to lessen the increased dietary Ca and Fe absorption that could otherwise result from hyperphagia. However, these effects are not likely to be mediated through increased enterocyte metallothionein, which appears not to bind to either Fe or Ca but operate through different, specific mechanisms.

Consistent with previous reports (38), mean serum ferritin was increased in diabetic subjects, although other measures of Fe homeostasis including serum Fe and IBC (39) were not different from control values. Basal urinary Fe excretion was also elevated in diabetic subjects, whereas fecal Fe and the Fe balance were similar. These observations could be explained by the increased metal uptake secondary to relative hyperphagia in the diabetic subjects before the imposition of strict dietary control during the controlled balance studies. Trientine's effects on uptake of Ca, Mn, and Se were similar to those for Fe. At present, however, the significance of high plasma ferritin concentrations in a subset of diabetic patients remains unclear (40), as does the mechanism of the negative correlation between ferritin and $[Cu]_{urine}$ observed here in trientine-treated diabetic subjects. In the Third National Health and Nutrition Examination Survey (1988–1994), an increased risk of diabetes was concentrated among participants with the lowest transferrin saturation concentrations; it was reported that this association was less likely to be explained by the Fe overload hypothesis and instead may be caused by inflammation as the mechanism by which ferritin becomes elevated in diabetes (38).

In our study we have shown that several alterations in the regulation of Cu homeostasis occur in diabetic humans. How might Cu and chronic hyperglycemia conspire to cause cardiovascular damage in diabetes? Ceruloplasmin and serum albumin are the main Cu-binding proteins in plasma (41), and there is some evidence that chronic hyperglycemia can damage the Cu-binding properties of both (42,43). Results from our recent studies have indicated that diabetes might cause two- to threefold increments in extracellular matrix Cu (8). Furthermore, incubation of ceruloplasmin with glucose reportedly causes fragmentation and time-dependent release of its bound Cu^{II}, which then appears to participate in a Fenton-type reaction to produce hydroxyl radicals (42). These data were interpreted as consistent with the conjecture

that ROS may form by the Maillard reaction and in turn generate hydroxyl radicals via a Cu-dependent Fenton-type reaction (42). In addition, glycated serum albumin reportedly becomes pro-oxidant in the presence of Cu, probably through the generation of ROS (44). Damage by chronic hyperglycemia to Cu-regulating mechanisms through glycation of ceruloplasmin and albumin could lead to elevated concentrations of catalytically active Cu^{II} in plasma, but this hypothesis requires support by direct observation. Cu binds to transferrin in addition to ceruloplasmin and albumin (45); this interaction could also be important in the association of Cu, EC-SOD, and HbA_{1c}.

In summary, the regulation of Cu homeostasis was altered in the diabetic subjects in this study, who demonstrated elevated rates of urinary Cu excretion and a tendency to increased Cu balance compared with control subjects. Treatment with the Cu^{II}-selective chelator, trientine, lowered Cu balance in the diabetic subjects, in whom elevated EC-SOD was strongly correlated with the $[Cu]_{plasma}$-HbA_{1c} interaction, implicating this in diabetic tissue damage. Trientine suppressed elevations of EC-SOD in diabetic subjects. In conjunction with therapies aimed at decreasing hyperglycemia, the use of compounds that can remove systemic Cu^{II} is proposed as a therapy for suppressing the tendency to cardiovascular complications in diabetes.

ACKNOWLEDGMENTS

This work was supported by the Endocore Research Trust and Protemix.

We thank T.B. Mulvey, G. Allen, S. Thornber, P. Misur, G. Muir, G. Kerr, G. Robinson, and P. Baker for assistance; and M.S. Cameron-Cooper, K.R. Mansford, P.J. Scott, T. Brittain, C.A. Tse, A.R. Phillips, P.D. Boyd, K. Wichmann, and D.J. Crossman for their discussions with us. G.C. acknowledges program support from the Foundation of Research, Science and Technology, New Zealand and the Health Research Council of New Zealand.

REFERENCES

1. Halliwell B, Gutteridge JM: *Free Radicals in Biology and Medicine.* 3rd ed. Oxford, Oxford University Press, 1999
2. Heart Protection Study Collaborative Group: MRC/BHF Heart Protection Study of antioxidant vitamin supplementation in 20,536 high-risk individuals: a randomised placebo-controlled trial. *Lancet* 360:23–33, 2002
3. Monnier VM: Transition metals redox: reviving an old plot for diabetic vascular disease. *J Clin Invest* 107:799–801, 2001
4. Fraústo da Silva JJ, Williams RJ: *The Biological Chemistry of the Elements: The Inorganic Chemistry of Life.* 2nd ed. Oxford, U.K., Clarendon Press, 2001
5. Wolff SP, Jiang ZY, Hunt JV: Protein glycation and oxidative stress in diabetes mellitus and aging. *Free Radic Biol Med* 10:339–352, 1991
6. Gu K, Cowie CC, Harris MI: Mortality in adults with and without diabetes in a national cohort of the U.S. population, 1971–1993. *Diabetes Care* 21:1138–1145, 1998
7. Struthers AD, Morris AD: Screening for and treating left-ventricular abnormalities in diabetes mellitus: a new way of reducing cardiac deaths. *Lancet* 359:1430–1432, 2002
8. Cooper GJ, Phillips AR, Choong SY, Leonard BL, Crossman DJ, Brunton DH, Saafi EL, Dissanayake AM, Cowan BR, Young AA, Occleshaw CJ, Chan YK, Leahy FE, Keogh GF, Gamble GD, Allen GR, Pope AJ, Boyd PD, Poppitt SD, Borg TK, Doughty RN, Baker JR: Regeneration of the heart in diabetes by selective copper chelation. *Diabetes* 53:2501–2508, 2004
9. Franz MJ, Bantle JP, Bebbe CA, Brunzell JD, Chaisson JL, Garg A, Holzmeister LA, Hoogwerf B, Mayer-David E, Mooradian AD, Purnell JQ, Wheeler M: Evidence-based nutrition principles and recommendations for the treatment and prevention of diabetes and related complications (Review). *Diabetes Care* 25:148–198, 2002
10. Clescerl LS, Greenberg AE, Eaton AD: *Standard Methods for the Examination of Water and Waste Water.* 20th ed. Washington, DC, American Public Health Association, 1998
11. Goldstein H: *Multilevel Statistical Models.* 3rd ed. London, U.K., Arnold, 2003
12. McCulloch CE, Searle SR: *Generalized, Linear, and Mixed Models.* New York, John Wiley, 2001
13. Kodama H, Murata Y, Iitsuka T, Abe T: Metabolism of administered triethylene tetramine dihydrochloride in humans. *Life Sci* 61:899–907, 1997
14. Smith RG, Heise CC, King JC, Costa FM, Kitzmiller JL: Serum and urinary magnesium, calcium and copper levels in insulin-dependent diabetic women. *J Trace Elem Electrolytes Health Dis* 2:239–243, 1988
15. Ito S, Fujita H, Narita T, Yaginuma T, Kawarada Y, Kawagoe N, Sugiyama T: Urinary copper excretion in type 2 diabetic patients with nephropathy. *Nephron* 88:307–312, 2001
16. Walter RM Jr, Uriu-Hare JY, Olin KL, Oster MH, Anawalt BD, Critchfield JW, Keen CL: Copper, zinc, manganese, and magnesium status and complications of diabetes mellitus. *Diabetes Care* 14:1050–1056, 1991
17. Hurrell RF, Ribas S, Davidsson L: NaFe3+EDTA as a food fortificant: influence on zinc, calcium and copper metabolism in the rat. *Br J Nutr* 71:85–93, 1994
18. Vachette P, Dainese E, Vasyliev VB, Di Muro P, Beltramini M, Svergun DI, De Filippis V, Salvato B: A key structural role for active site type 3 copper ions in human ceruloplasmin. *J Biol Chem* 277:40823–40831, 2002
19. Hellman NE, Gitlin JD: Ceruloplasmin metabolism and function. *Annu Rev Nutr* 22:439–458, 2002
20. Kehoe CA, Faughman MS, Gilmore WS, Coulter JS, Howard AN, Strain JJ: Plasma diamine oxidase is greater in copper-adequate than copper-marginal or copper-deficient rats. *J Nutr* 130:30–33, 2000
21. Hambidge M: Biomarkers of trace mineral intake and status. *J Nutr* 133:948S–955S, 2003
22. Milne DB, Nielsen FH: Effects of a diet low in copper on copper-status indicators in postmenopausal women. *Am J Clin Nutr* 63:358–364, 1996
23. Peña MM, Lee J, Thiele DJ: A delicate balance: homeostatic control of copper uptake and distribution. *J Nutr* 129:1251–1260, 1999
24. Kadiiska MB, Hanna PM, Hernandez L, Mason RP: In vivo evidence of hydroxyl radical formation after acute copper and ascorbic acid intake: electron spin resonance spin-trapping investigation. *Mol Pharmacol* 42: 723–729, 1992
25. Diabetes Control and Complications Trial Research Group: The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med* 329:977–986, 1993
26. Adachi T, Nakamura M, Yamada H, Futenma A, Kato K, Hirano K: Quantitative and qualitative changes of extracellular-superoxide dismutase in patients with various diseases. *Clin Chim Acta* 229:123–131, 1994
27. Fukai T, Folz RJ, Landmesser U, Harrison DG: Extracellular superoxide dismutase and cardiovascular disease. *Cardiovasc Res* 55:239–249, 2002
28. Adachi T, Inoue M, Hara H, Maehata E, Suzuki S: Relationship of plasma extracellular-superoxide dismutase level with insulin resistance in type 2 diabetic patients. *J Endocrinol* 181:413–417, 2004
29. Kimura F, Hasegawa G, Obayashi H, Adachi T, Hara H, Ohta M, Fukui M, Kitagawa Y, Park H, Nakamura N, Nakano K, Yoshikawa T: Serum extracellular superoxide dismutase in patients with type 2 diabetes: relationship to the development of micro- and macrovascular complications. *Diabetes Care* 26:1246–1250, 2003
30. Vivoli G, Bergomi M, Rovesti S, Pinotti M, Caselgrandi E: Zinc, copper, and zinc- or copper-dependent enzymes in human hypertension. *Biol Trace Elem Res* 49:97–106, 1995
31. Lloyd-Jones DM, Bloch KD: The vascular biology of nitric oxide and its role in atherogenesis. *Annu Rev Med* 47:365–375, 1996
32. Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Brownlee M: Normalizing mitochondrial superoxide production blocks three pathways of hyperglycemic damage. *Nature* 404:787–791, 2000
33. Panel on Micronutrients, Subcommittees on Upper Reference Levels of Nutrients and of Interpretation and Use of Dietary Reference Intakes, and the Standing Committee on the Scientific Evaluation of Dietary Reference Intakes: *Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc.* Washington, DC, National Academy of Sciences, 2001
34. Panel on Dietary Antioxidants and Related Compounds, Subcommittees on Upper Reference Levels of Nutrients and Interpretation and Uses of

DRIs, Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, Food and Nutrition Board: *Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids.* Washington, DC, National Academy of Sciences, 2000
35. Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, Food and Nutrition Board, Institute of Medicine: *Dietary Reference Intakes for Calcium, Phosphorus, Magnesium, Vitamin D, and Fluoride.* Washington, DC, National Academy of Sciences, 1997
36. Escobar O, Sandoval M, Vargas A, Hempe JM: Role of metallothionein and cysteine-rich intestinal protein in regulation of zinc absorption by diabetic rats. *Pediatr Res* 37:321–327, 1995
37. Keen CL, Cohen NL, Lonnerdal B, Hurley LS: Teratogenesis and low copper status resulting from triethylenetetramine in rats. *Proc Soc Exp Biol Med* 173:598–605, 1983
38. Ford ES, Cogswell ME: Diabetes and serum ferritin concentration among U.S. adults. *Diabetes Care* 22:1978–1983, 1999
39. Cutler P: Deferoxamine therapy in high-ferritin diabetes. *Diabetes* 38:1207–1210, 1989
40. Van Oost BA, Van den Beld B, Cloin LG, Marx JJ: Measurement of ferritin in serum: application in diagnostic use. *Clin Biochem* 17:263–269, 1984
41. Linder MC: *Biochemistry of Copper.* New York, Plenum, 1991
42. Islam KN, Takahashi M, Higashiyama S, Myint T, Uozumi N, Hayanoki Y, Kaneto H, Kisaka H, Taniguchi N: Fragmentation of ceruloplasmin following nonenzymatic glycation reaction. *J Biochem* 118:1054–1060, 1995
43. Argirova MD, Ortwerth BJ: Activation of protein-bound copper ions during early glycation: study on two proteins. *Arch Biochem Biophys* 420:176–184, 2003
44. Bourdon E, Loreau N, Blache D: Glucose and free radicals impair the antioxidant properties of serum albumin. *FASEB J* 13:233–244, 1999
45. Hirose J, Fujiwara H, Magarifuchi T, Iguti Y, Iwamoto H, Kominami S, Hiromi K: Copper binding selectivity of N and C sites in serum and ovotransferrin. *Biochim Biophys Acta* 1296:103–111, 1996