Elevated Plasma Ceruloplasmin in Insulin-Dependent Diabetes Mellitus: Evidence for Increased Oxidative Stress as a Variable Complication

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Ceruloplasmin (Cp) is an acute-phase–responsive oxidase enzyme. Prior reports suggest that Cp is increased in diabetes mellitus, perhaps reflecting greater oxidant stress. However, the situation in insulin-dependent diabetes mellitus (IDDM) per se remains unclear. Furthermore, vitamin C can interfere with one indirect assay for Cp, and vitamin C metabolism is altered in IDDM. We measured Cp levels by both a direct radial immunodiffusion (RID) assay and an indirect oxidase assay in 10 subjects with IDDM and 10 nondiabetics, both at baseline and after 30 days of vitamin C supplementation (100 or 600 mg daily, five subjects per group). Plasma copper level was measured independently also. Our data show that circulating levels of Cp are significantly increased in IDDM subjects as a group, and specifically that Cp is abnormally high in a subset of IDDM individuals. Vitamin C supplementation at either dose interfered with the oxidase assay for Cp in both groups, but vitamin C did not alter the RID assay. The observed increase in plasma copper suggests that circulating holo-Cp is increased. The finding of increased Cp in some individuals with IDDM supports the hypothesis of increased oxidant stress as a variable factor in the spectrum of chronic complications in diabetes. Measurements of Cp level by the oxidase assay must be considered unreliable for subjects taking vitamin C supplements of ≥100 mg/d.

CERULOPLASMIN (Cp) is a circulating cuproprotein with well-characterized functions in the metabolism of copper and iron.[1] Holo-Cp contains six atoms of copper per mole and transports approximately 80% of the copper present in plasma.[2] In addition to its role in copper transport, Cp functions as the "ferroxidase" required for mobilization of iron from enterocytes. Oxygen is directly reduced to water in that redox reaction,[3] and may also represent the mechanism by which Cp inhibits superoxide-induced lipid peroxidation.[4] Therefore, elevated plasma Cp levels could signal abnormally high oxidant stress.

Increased oxidant stress has been implicated in the pathogenesis of diabetes mellitus. Hyperglycemia-induced protein glycosylation generates superoxide free radicals.[5] Plasma peroxide levels are higher in diabetics than in nondiabetics.[6,7] A scenario of unusually high oxidant stress in diabetes is corroborated by a report[8] documenting increased circulating levels of acute-phase–responsive proteins, including Cp, in a series consisting primarily of subjects with non–insulin-dependent diabetes mellitus (NIDDM).

Many investigators have measured circulating levels of copper, Cp, or both in diabetes mellitus.[7-20] Only a few of these studies report data specifically for individuals with insulin-dependent diabetes mellitus,[9-11] whereas the remainder include either combined IDDM and NIDDM subjects or undefined diabetic groups. Plasma copper is increased in IDDM,[9,10] in agreement with some studies of NIDDM subjects or mixed diabetics.[7,12,20] However, other studies report normal copper levels[13,18] in the latter groups. Circulating Cp level is most often reported using an indirect oxidase-activity assay.[7,11,14-19] These studies report that Cp is either significantly increased,[14-16] borderline increased,[7] or normal[17-19] in NIDDM subjects or mixed diabetics. The single report examining IDDM finds Cp levels to be decreased.[11] The direct assay of Cp by radial immunodiffusion (RID) has not been reported for IDDM, but is either increased[8] or normal[20] in mixed diabetes or NIDDM groups.

An increase in circulating Cp in IDDM per se, if documented by RID, could support the notion of a heightened acute-phase physiology in diabetes.[8,14] We now report measurements of Cp levels, both by direct RID assay and by indirect oxidase assay, in young adults with IDDM and matched nondiabetic controls. We also explored whether the known interference by vitamin C in the oxidase assay for Cp[21] could reflect the vitamin's role in removing copper from Cp.[22] Apo-Cp in circulation has a shortened half-life.[23] To investigate this possibility, both groups were studied again after 30 days of supplemental vitamin C (100 or 600 mg daily).

SUBJECTS AND METHODS

This study was conducted as a component of a protocol examining the effect of vitamin C supplementation on erythrocyte sorbitol concentrations that is reported elsewhere.[24] Young adult volunteers were recruited by print advertisement on our university campus. Participation was restricted to those for whom a medical record could be kept at the University Health Service. Ten young adults with IDDM (five women and five men) aged 19 to 34 years whose disease was diagnosed 6 to 23 years before study were recruited as volunteers. One of these, a woman in the 600-mg supplement group, was not included in the parallel report on sorbitol[24] because she did not complete the second month of that protocol. Ten nondiabetic young adults (five women and five men) aged 23 to 35 years also participated. An eleventh nondiabetic male subject who was included in the sorbitol study[24] is not reported herein due to insufficient sample volumes for the present assays. The protocol was approved by the Research Committee of the University Health Service. All subjects were nonsmokers and were

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not obese. Individuals with IDDM were in variable glycemic control, as evidenced by baseline glycosylated hemoglobin levels of 6% to 15% and 24-hour glucosuria levels ranging across three orders of magnitude from 3 to 1,890 mmol/mol creatinine.[24]

Subjects maintained their habitual dietary pattern supplemented once daily with ascorbic acid in tablet form at a dose of either 100 mg (0.57 mmol, five from each group) or 600 mg (3.4 mmol, five from each group). Food intake records were kept for 3 consecutive days between days 0 and 3 (baseline) and days 28 and 34 (termed day 30). These were analyzed using our Massachusetts Nutrient Data Bank facility. A venous blood sample was collected by a phlebotomist at the University Health Service on day 0 and again on day 30. Plasma was assayed for ascorbic acid by a standard spectrophotometric assay.[25] Cp was assayed by oxidase activity using *p*-phenylamine diamine (PPD) as the substrate[26] and also by a commercial RID assay kit (Norpartigen kit; Behring Diagnostics, Somerville, NJ). PPD is washed and recrystallized before use, narrowing the normal range for Cp to 23 to 43 mg/dL in the PPD assay in our hands (published range of the assay by the Clinical Chemistry Department, Massachusetts General Hospital). Plasma Cu was assayed by atomic absorption spectrometry (AAS) using a Perkin-Elmer Model 2380 AAS unit (Norwalk, CT) fitted with a single-element cathode source and set for 324-nm detection with a 0.7-nm slit width. Appropriate standards were prepared for the assay. We calculated the ratio of copper to Cp in plasma, defined as ratio = [1,000 × copper (μg/dL)]/Cp(mg/dL). The expected ratio in holo-Cp is 3.0 if one presumes circulating Cp to weigh 132 kd and to contain six Cu atoms per mole.[27,28]

Statistical analyses included *t* tests and 2 × 2 factorial ANOVAs. Raw data were analyzed when Bartlett's test for homogeneity of variances was nonsignificant. Otherwise, transformed data (natural logarithm or square root) satisfying this test were analyzed. Main effects in ANOVA were the group (IDDM *v* nondiabetic) and supplementation (day 0 *v* day 30). Tukey's post hoc mean comparison test was applied when ANOVAs were significant. A commercial software program was used (Systat, Evanston, IL). All data are reported as the mean ± SEM.

RESULTS

Baseline Comparisons

We now report for the first time in IDDM per se that plasma Cp is significantly increased and that the increase is seen when assayed by either the RID or oxidase (PPD) methods, as shown in Fig 1. Using RID data (Fig 1A), all nondiabetics had levels less than our maximal normal level of 43 mg/dL (range, 20.3 to 40.2). However, Cp exceeded this upper limit of normal in four of 10 IDDM subjects (two men and two women), and no IDDM subject had a level less than 30 mg/dL (overall range, 30.9 to 58.2). Neither the duration of IDDM nor the degree of glycemic control, as judged by glycosylated hemoglobin and plasma glucose, were different between those IDDM individuals with elevated Cp and the remainder of IDDM subjects (data not shown). The general elevation of Cp in the IDDM group is consistent with the significantly ($P < .001$) increased plasma copper that was measured independently by AAS (Table 1).

Supplementation Effects

Dietary intakes of vitamin C continued as usual, as judged by 3-day food records. As expected, plasma ascorbic acid concentrations were increased on day 30 by approximately 50%, reaching 104 ± 5.1 μmol/L in IDDM subjects and 122 ± 7.5 in nondiabetics (P = NS). Supplement level was not a significant factor for any variable studied, and so the data were combined within each group for ANOVA comparisons (group, sex, and supplement as main effects with their paired interactions).

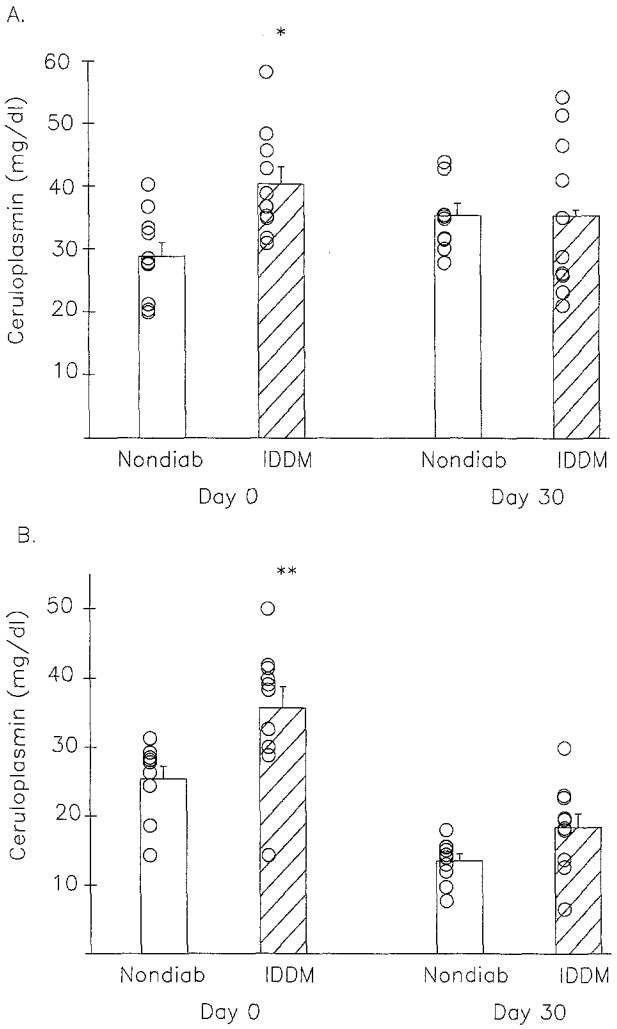

Fig 1. Plasma Cp levels measured by RID (A) or by PPD assay (B) during vitamin C supplementation of 100 (5 from each group) or 600 mg/d (5 from each group) between baseline and day 30 (level nonsignificant, data combined). (A) 2 × 2 ANOVA for RID assay: IDDM *v* nondiabetic ($P < .05$), vitamin C (NS). (B) 2 × 2 ANOVA for PPD assay: IDDM *v* nondiabetic ($P < .01$), vitamin C ($P < .001$). *$P < .05$ *v* nondiabetic by Tukey's post hoc analysis. **$P < .01$ *v* nondiabetic by Tukey's post hoc analysis.

Levels of Cp obtained after vitamin C supplementation are depicted in Fig 1 for each assay. Cp is unchanged by the direct RID assay (Fig 1A) in both groups (P = NS for supplementation effect). Among four individuals with abnormally elevated baseline Cp, three remain elevated. In contrast, the indirect oxidase assay using PPD (Fig 1B) shows Cp to be significantly decreased ($P < .0001$) in all subjects at day 30. PPD data were transformed before ANOVA to correct for a lack of homogeneity among variances. Cp level is less than normal in all 10 nondiabetics and in seven of 10 IDDM subjects. Cp level is no longer

Table 1. Plasma Copper Levels Measured by AAS and the Ratio of Copper to Cp in Plasma

Parameter	Women	Men	2 × 2 ANOVA†	
			Plasma Copper	Copper to Cp Ratio
Plasma copper (μg/dL)				
IDDM subjects	133 ± 4.9	98 ± 4.2		
Nondiabetics	89 ± 3.6	88 ± 3.0		
Plasma copper to Cp ratio*				
IDDM subjects	3.4 ± 0.35	3.0 ± 0.17		
Nondiabetics	3.1 ± 0.26	2.9 ± 0.14		
Group			$P < .001$	P = NS
Sex			$P < .001$	P = NS
Group × sex			$P < .005$	P = NS

NOTE. Data summarize 10 measurements in each group (pairs at baseline and day 30 for 5 subjects per category). The effect of vitamin C level (100 mg/d in 5 from each group or 600 mg/d in 5 from each group) was nonsignificant. The effect for vitamin C (baseline *v* day 30, 10 in each group) was also not significant in 2 × 2 × 2 ANOVAs.

*Ratio = plasma copper level measured by AAS divided by Cp level measured using the RID assay.

†Data transformed by log or square root to satisfy Bartlett's test.

significantly higher in IDDM subjects than in nondiabetics by Tukey's post hoc mean comparison test. No differences in Cp due to sex were found for either assay.

Plasma levels of copper remained steady at day 30 (Table 1), so that circulating copper levels in IDDM subjects continued to be greater than in nondiabetics, both within each sex and overall. Again, data were transformed before ANOVAs to satisfy homogeneity. The effect of vitamin C supplementation was not significant, and as a result the means in Table 1 are derived from paired measurements for each of five individuals per cell. Significant effects of both group ($P < .001$) and sex ($P < .001$) and a significant ($P < .01$) interaction effect are present using a 2 × 2 ANOVA. This interaction signifies that copper levels in diabetic males are intermediate between those of diabetic females and nondiabetic females, and that nondiabetic men have the lowest copper levels (Table 1).

The ratios of copper to Cp (by RID) in plasma are also listed in Table 1. These ratios, although variable, are consistent with normal copper physiology for each sex in both groups at both times (main effects on transformed data all NS by 2 × 2 × 2 ANOVA). The correlation between Cp and plasma copper is significant overall ($N = 40$, $r = .58, P < .001$).

DISCUSSION

Circulating Cp and Copper Status in IDDM

The direct RID assay appears to be the criterion assay of choice for Cp (see later discussion). We show that circulating Cp is frequently increased above normal in free-living individuals with IDDM. This is in disagreement with a prior report of decreased Cp levels in IDDM subjects as a group,[11] in which the indirect oxidase assay was used. That finding itself stands in singular contrast to the many studies reporting circulating levels of Cp to be increased[8,14-16] or at least normal[7,17-20] in NIDDM or mixed diabetic groups. Walter et al[7] found no differences in Cp by type of diabetes. They and others[7,9,10] report increased plasma copper levels in IDDM, suggesting that our data are correct. Furthermore, our finding is independently supported by the increased plasma copper level measured by AAS. An increase in Cp in a subgroup of individuals with IDDM is consistent with the notion that a state of heightened oxidative stress may occur. However, it is important to note that our findings show elevated Cp not to be a universal state in IDDM, such as is the case for direct physiologic alterations including increased zincuria,[27] but rather presents as a variable finding, as expected for most of the chronic degenerative syndromes associated with diabetes.

The effect of vitamin C supplementation on copper physiology in IDDM is difficult to interpret from our data. Total intakes of vitamin C, the sum of the dietary intake plus the assigned supplement, averaged 195 mg/d for all subjects on the low-dose supplement, versus 760 mg/d for those on the higher dose, and did not differ by group within either supplement.[24] Elevated plasma levels of ascorbate, an antioxidant, would be expected to lessen oxidative stress. Circulating Cp (RID assay) did decrease and normalize for IDDM subjects as a group, but the abnormally elevated Cp remained in three of four individual subjects. In addition, plasma copper remained elevated in IDDM subjects (Table 1). Unfortunately, the calculation of copper to Cp ratios does not clarify these data, since they varied considerably around the theoretic value[28] of 3.0. It is therefore not possible to draw a definitive conclusion from our presently limited data regarding the amelioration of an oxidative-stress milieu in IDDM by supplemental vitamin C.

Cp Assay During Vitamin C Supplementation

The present study involved moderate supplements of vitamin C in young nonsmoking adults. One study of young men taking substantially more vitamin C (1,500 mg daily) for 64 days[29] reported a significant decrease in Cp using the PPD oxidase assay. It could be postulated that the vitamin C–dependent copper uptake from Cp[22] is enhanced during this supplementation, with the resulting apo-Cp being preferentially cleared from plasma.[23] Subsequently, a definitive study by Jacob et al[21] demonstrated that vitamin C supplementation (605 mg/d) did decrease Cp levels when measured by the indirect oxidase assay, but that Cp levels measured by the direct RID assay and plasma copper levels by AAS did not change. This appears to negate the enhanced-transport and apo-Cp postulate. We now confirm these latter observations in nondiabetics and extend them both to lower-dose supplements of vitamin C (100 mg) and to individuals with IDDM (Fig 1). An elevation of plasma ascorbic acid in vivo to greater than 100 μmol/L appears to be sufficient for interference with the oxidase assay for Cp. In vitro replication of the effect by spiking plasma with ascorbic acid requires a 10-fold higher level.[21] Our direct assay of copper in plasma (Table 1) confirms the artifactual nature of the oxidase results. The calculated copper to Cp ratios, although variable, are at least as high as expected[28]

and thereby serve to eliminate the apo-Cp premise for the mechanism of vitamin C interference. We conclude that the oxidase (PPD) assay is unreliable for monitoring copper status in individuals with vitamin C intakes sufficient to elevate plasma ascorbic acid levels to greater than 100 μmol/L. The RID assay for Cp was not altered by vitamin C supplementation and remained appropriate for plasma copper, as indicated by copper to Cp ratios.

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