Common Presence of Non–Transferrin-Bound Iron Among Patients With Type 2 Diabetes

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OBJECTIVE — Recently, we reported increased cardiovascular disease mortality among supplemental vitamin C users with type 2 diabetes in a prospective cohort study. Because vitamin C may cause oxidative stress in the presence of redox active iron, we hypothesized that non–transferrin-bound iron (NTBI), a form of iron susceptible to redox activity, may be present in patients with type 2 diabetes.

RESEARCH DESIGN AND METHODS — We measured serum NTBI levels using high-performance liquid chromatography in 48 patients with known diabetes (at least 5 years duration since diagnosis), 49 patients with newly diagnosed diabetes, and 47 healthy control subjects (frequency matched on age and sex).

RESULTS — NTBI was commonly present in diabetes: 59% in newly diagnosed diabetes and 92% in advanced diabetes. Mean NTBI values varied significantly between the three groups, with the highest values being observed in patients with known diabetes and the lowest in the control subjects (0.62 ± 0.43 vs. 0.24 ± 0.29 vs. 0.04 ± 0.13 μmol/l Fe). Serum total iron or percent transferrin saturation were very similar among the three groups, yet NTBI was strongly associated with serum total iron ($r = 0.74$, $P < 0.01$) and percent transferrin saturation ($r = 0.70$, $P < 0.01$) among the patients with known diabetes.

CONCLUSIONS — Consistent with our hypothesis, these data demonstrate the common existence of NTBI in type 2 diabetic patients with a strong gradient with severity. Prospective cohort studies are required to clarify the clinical relevance of increased NTBI levels.

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Recently, we reported that supplemental vitamin C intake in excess of 300 mg/day increased cardiovascular disease (CVD) mortality among postmenopausal women with type 2 diabetes in a prospective cohort study (1). This finding was unexpected in that it is contrary to the general belief that supplemental vitamin C intake is beneficial for patients with type 2 diabetes because they tend to have lower serum vitamin C levels (2,3). One explanation for this finding is that vitamin C may interact with redox active iron in patients with type 2 diabetes (4,5).

Although the prooxidant reaction of vitamin C occurs readily in vitro, its relevance in vivo has been a matter of some controversy, the main point of contention being the availability of redox active iron in vivo (6). Levels of redox active iron are thought to be low due to their sequestration by various metal-binding proteins such as transferrin (7). It is generally believed that non–transferrin-bound iron (NTBI) possesses redox activity but only appears in serum when transferrin is fully iron saturated (8). However, recent studies have demonstrated that NTBI can be detected when transferrin is not fully saturated, leading to a revision of the original understanding that NTBI results from a simple spillover phenomenon (9–11). Interestingly, a disturbance of iron metabolism and/or iron overload has recently been suggested as a mechanism of the pathogenesis of both diabetes and CVD complications (12,13).

The aim of this study was to establish whether NTBI is present in patients with type 2 diabetes and whether there is a relationship between NTBI and common iron-related biomarkers or other biochemical variables.

RESEARCH DESIGN AND METHODS

We selected 49 patients with newly diagnosed type 2 diabetes together with a group of 47 age and sex frequency-matched control subjects through a community-based health examination performed between June 2003 and August 2003 in Haman, Korea. In addition, 48 age and sex frequency-matched patients with known type 2 diabetes who had diabetes for at least 5 years were selected from outpatients of the endocrinology department in our university-affiliated hospital between January 2004 and March 2004. The mean ± SD duration subsequent to type 2 diabetes diagnosis among those with known diabetes was 9.7 ± 5.8 years, and 95% were treated with oral agents. After a 12-h overnight fast, blood samples were collected via an antecubital vein and sera were separated and stored at −70°C for all analyses.

NTBI measurement

NTBI measurement was undertaken using a previously reported high-performance liquid chromatography (HPLC) method (14) with appropriate

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Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; CVD, cardiovascular disease; GGT, γ-glutamyl transferase; HPLC, high-performance liquid chromatography; NTBI, non–transferrin-bound iron.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

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Table 1—*Distribution of demographic and clinical variables among healthy control, newly diagnosed type 2 diabetic, and known type 2 diabetic participants*

	Control	Newly diagnosed diabetes	Known diabetes	*P*
n	47	49	48	
Age (years)	58.3 ± 9.7	59.8 ± 9.7	58.9 ± 9.2	0.74
Men (%)	72.3	71.4	62.5	0.64
Current smoker (%)	31.9	38.8	39.6	0.70
Alcohol user (≥1 unit/week) (%)	31.9	42.9	18.8‡	0.04
Fasting blood glucose (mg/dl)	87.7 ± 7.9	153.4 ± 38.6*	136.8 ± 36.8†‡	<0.01
A1C (%)	—	—	8.1 ± 1.7	
CRP (mg/l)§	1.2 ± 1.3	1.7 ± 2.1	3.1 ± 4.6†‡	<0.01
Iron (μg/dl)§	95.5 ± 41.9	120.1 ± 53.9	101.2 ± 33.3	0.29
Ferritin (ng/ml)§	83.2 ± 56.7	137.0 ± 90.9	174.5 ± 137.4†	<0.01
Transferrin saturation (%)§	32.0 ± 15.4	29.4 ± 11.7	33.0 ± 11.2	0.23
Hemoglobin (g/dl)	13.1 ± 1.2	13.2 ± 1.6	14.2 ± 1.5†‡	<0.01
GGT (units/l)	13.3 ± 5.3	50.6 ± 55.3*	70.7 ± 66.5†‡	<0.01
ALT (units/l)	13.3 ± 8.2	30.0 ± 27.8*	34.6 ± 22.6†	<0.01
AST (units/l)	19.5 ± 7.0	27.3 ± 13.8*	28.6 ± 12.6†	<0.01

Data are arithmetic means ± SD or proportion. *P* values were based on one-way ANOVA, and multiple comparisons were performed by Scheffe's test: *control vs. newly diagnosed diabetes; †control vs. known diabetes; ‡newly diagnosed diabetes vs. known diabetes. All serum variables, except hemoglobin, were transformed using the natural logarithm before ANOVA, and multiple comparisons were performed. §Iron, ferritin, percent transferrin saturation, and CRP were measured only among 116 study subjects (33 in the control, 36 in the newly diagnosed diabetes, and 47 in the known diabetes groups).

modifications. The samples from patients with newly diagnosed diabetes were analyzed in December 2003, and those from control and advanced diabetes groups were analyzed in June 2005. Sodium triscarbonatocobaltate(III) trihydrate $\{Na_3[Co(CO_3)_3]\cdot 0.3\ H_2O\}$ solution containing 4.5 mmol/l Co(III) in 1 mol/l sodium bicarbonate was prepared as previously described (14), and 50 μl was added to serum (250 μl) followed by incubation at 37°C for 1 h. The samples were subsequently added to nitriltriacetic acid solution (30 μl, 0.8 mol/l, pH 7.0) and permitted to stand at room temperature for 30 min. The samples were ultrafiltered using a centrifugal filter device (Microcon YM-30, 30,000 MWCO; Millipore, County Cork, Ireland) at 14,000 rpm for 30 min. The filtrates (20 μl) were directly subjected to HPLC analysis.

The metal-free HPLC system used in the investigation consisted of a 996 photodiode array detector, a 600S controller, a 626 pump, and a 717 plus Autosampler (Waters). The system was controlled using chromatography software Millenium32, version 3.20. The column used for analysis was PLRP-S 100 Å (150 × 4.6 mm, 5 μm; Polymer Laboratories, Church Stretton, Shropshire, U.K.). The mobile phase consisted of 1,2-dimethyl-3-hydroxy-4(1H)-pyridinone (Deferiprone, CP20, L1) (3 mmol/l) in morpholinopropanesulfonic acid buffer (5 mmol/l, pH 7.0) and 20% acetonitrile (vol/vol). The flow rate was 1.0 ml/min, and the $Fe(CP20)_3$ complex, which was formed on column, was monitored at 450 nm. All measurements were performed in duplicate.

Previously it had been noted that, using the above HPLC method, negative NTBI values result from sera from healthy normal people, in whom NTBI is absent (14). The use of the cobalt solution in the sample preparation blocked up to 80% of the vacant transferrin sites, thus greatly reducing the underestimation of NTBI compared with the analysis in the absence of cobalt solutions. However, because cobalt cannot completely block the vacant sites of the unsaturated transferrin, the remaining unblocked sites are available for Fe(III)-introduced samples when cobalt and nitriltriacetic acid solutions are added to samples. Negative values arise because it is impossible to use normal serum for the preparation of calibration standard samples, and there is a consequent difference between samples and standards. Thus, all negative values can best be simply interpreted as NTBI being absent from the sample. For this study, all negative values of NTBI were set to 0.

Biochemical measurements

Serum iron, iron-binding capacity, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and γ-glutamyl transferase (GGT) were analyzed by colorimetric methods, and high-sensitivity C-reactive protein (CRP) was analyzed by an immunoturbidimetric method with a Hitachi 747 autoanalyzer (Hitachi, Tokyo, Japan). Serum ferritin was measured by a microparticle enzyme immunoassay with an Axsym System (Abbott Laboratories, Abbott Park, IL). Hemoglobin was measured with an ADVIA 120 Hematology System (Bayer HealthCare, Tarrytown, NY). HbA_{1c} (A1C) was measured by ion-change HPCL by the Bio-Rad D-10 hemoglobin testing system (Bio-Rad Laboratories, Hercules, CA).

Statistical analysis

Differences between means were analyzed using one-way ANOVA. Variables with a right-skewed distribution were transformed using the natural logarithm. Multiple comparison testing was performed by Scheffe's test. Pearson correlation coefficients were computed between NTBI and other biochemical variables. All statistical analyses were performed using SPSS 12.0.

RESULTS — Table 1 shows the distribution of demographic and clinical variables among the control and the two patient groups. Analysis of conventional iron markers demonstrated that only serum ferritin levels were significantly higher in the known diabetes group than

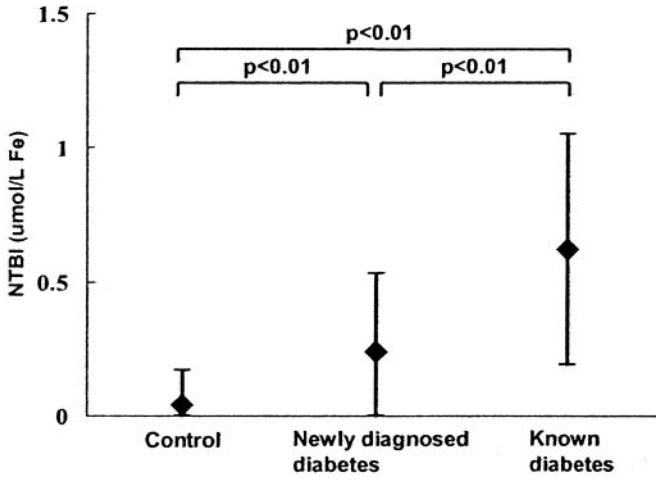

Figure 1—*Mean ± SD NTBI among control (0.04 ± 0.13), newly diagnosed diabetes (0.24 ± 0.29), and known diabetes (0.62 ± 0.43) groups.*

in the control group. There were no significant differences in either the serum iron or percent transferrin saturation across the three groups. GGT levels were significantly different in the three groups, whereas serum ALT and AST levels were significantly higher in both known diabetes and recently diagnosed diabetes groups when compared with the control group. Serum CRP and hemoglobin levels were significantly higher in the known diabetes group compared with both the newly diagnosed diabetes and control groups.

The mean values of NTBI for the three groups were significantly different from each other, with the highest levels being found in the known diabetes group and the lowest in the control subjects (0.62 ± 0.43 vs. 0.24 ± 0.29 vs. 0.04 ± 0.13 μmol/l Fe) (Fig. 1). NTBI was detected in 91.7% of the patients with known diabetes, 59.2% of patients with newly diagnosed diabetes, and 10.6% of the control subjects.

When the three groups were analyzed separately, NTBI was differently associated with biochemical variables by group (Table 2 and Fig. 2). In the control group, no variable was significantly associated with NTBI. Among those with newly diagnosed diabetes, NTBI levels appeared to be more strongly associated with liver enzymes such as ALT ($r = 0.30$, $P = 0.03$), AST ($r = 0.42$, $P < 0.01$), and GGT ($r = 0.45$, $P < 0.01$) than the conventional iron markers (Table 2). In those with known diabetes, NTBI was mostly positively associated with conventional iron markers; thus NTBI was strongly associated with percent transferrin saturation ($r = 0.70$, $P < 0.01$) (Fig. 1*D*) and serum total iron ($r = 0.74$, $P < 0.01$) (Fig. 1*F*) but weakly associated with ferritin ($r = 0.30$, $P = 0.04$) (Fig. 1*E*). On the other hand, serum CRP showed a significant positive association with NTBI among patients with newly diagnosed diabetes but a significant inverse association with NTBI among patients with known diabetes. A1C showed a nonsignificant inverse association with NTBI among those with known diabetes.

CONCLUSIONS— The present study was motivated by our previous finding that supplemental vitamin C intake increased the risk of CVD in type 2 diabetic patients (1). It is well known that vitamin C can act as a prooxidant under the presence of redox active iron (4,5). Therefore, we established a general hypothesis that redox active iron in diabetes could be involved in the increased CVD risk with supplemental vitamin C among type 2 diabetic subjects. So, as a first step in the investigation of the general hypothesis, we assayed NTBI in diabetic and nondiabetic subjects. In this study, NTBI was found to commonly exist in patients with type 2 diabetes, with a dose response related to the severity of diabetes. To the best of our knowledge, this is the first study reporting that the presence of NTBI is common to type 2 diabetic patients.

The exact nature of NTBI is still unclear, but it is believed to consist of iron loosely complexed to plasma components such as albumin, citrate, and phosphate. Unlike tightly bound storage and transport protein iron such as transferrin and ferritin, NTBI is a source of redox active iron (15). NTBI was initially reported in patients with severe iron overload, conditions associated with 100% saturation of transferrin such as thalassemia major (8). More recently, NTBI has been detected in patients with transferrin saturation falling within the normal range, such as alcohol

Table 2—*Pearson correlation coefficients* between NTBI and other clinical variables among control, newly diagnosed diabetes, or known diabetes groups*

	Control		Newly diagnosed diabetes		Known diabetes	
	r	*P*	*r*	*P*	*r*	*P*
Transferrin saturation (%)	−0.07	0.71	0.24	0.16	0.70	<0.01
Ferritin (ng/ml)	−0.18	0.33	0.13	0.47	0.30	0.04
Iron (μg/dl)	−0.11	0.53	0.27	0.11	0.74	<0.01
Hemoglobin (mg/dl)	−0.26	0.07	0.22	0.14	0.37	<0.01
GGT (units/l)	−0.05	0.75	0.45	<0.01	0.18	0.23
ALT (units/l)	+0.07	0.64	0.30	0.03	0.08	0.61
AST (units/l)	−0.08	0.60	0.42	<0.01	0.15	0.31
CRP (mg/l)	−0.13	0.49	0.38	0.02	−0.35	0.02
A1C (%)					−0.28	0.06

*All variables, except NTBI, hemoglobin, and A1C, were transformed using the natural logarithm before correlation coefficients were calculated.

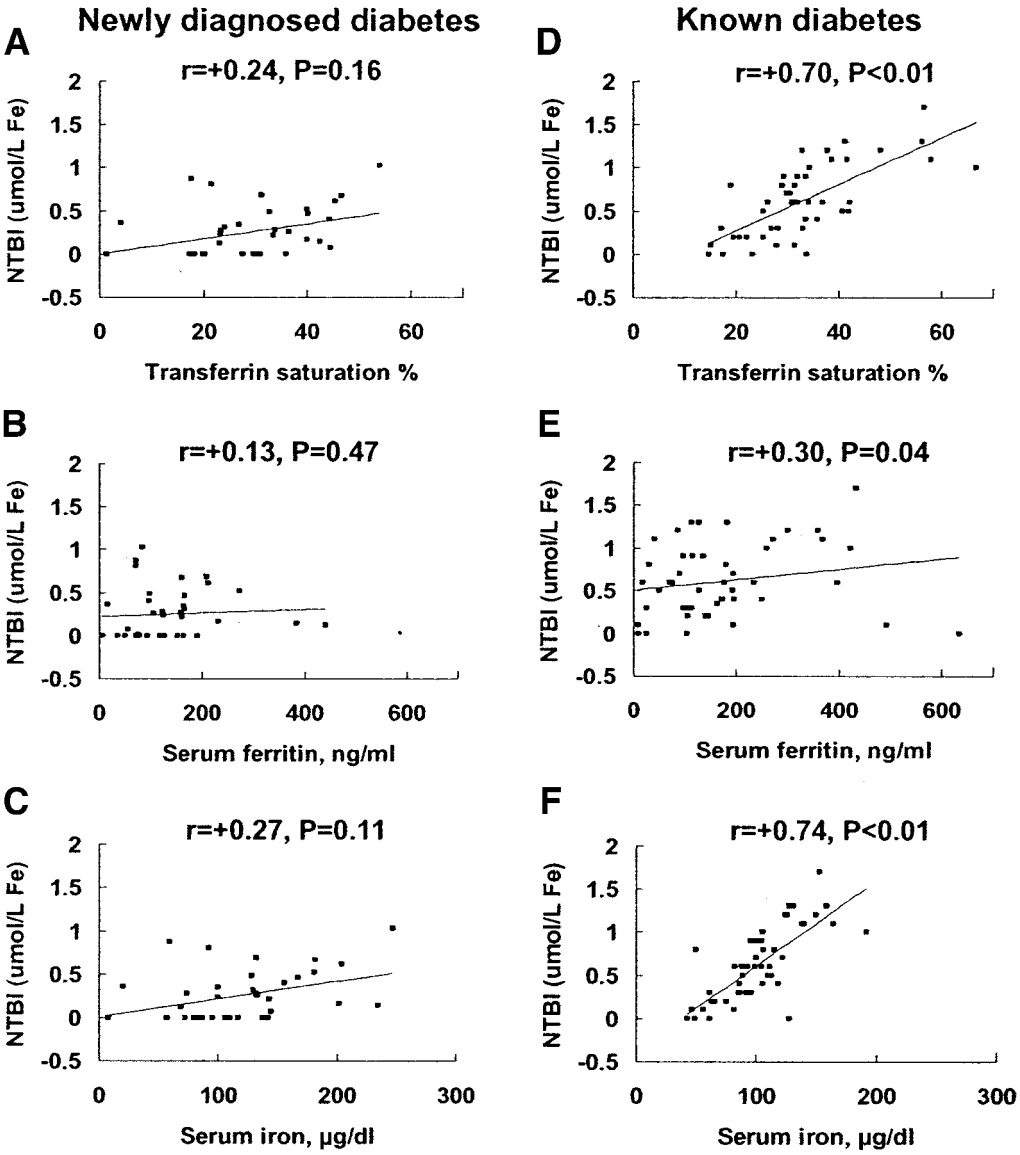

Figure 2—*Scatter diagrams between NTBI and percent serum transferrin saturation, ferritin, or iron among newly diagnosed diabetes* (left panel: A, B, *and* C) *or known diabetes* (right panel: D, E, *and* F) *groups.*

abusers and heterozygous hemochromatosis patients (9–11).

In this study, only six subjects had percent transferrin saturation values exceeding 50%, with a maximum of 67%. However, NTBI was detected in 59% of the patients with newly diagnosed diabetes and 92% of the patients with known diabetes. This finding supports the concept that full saturation of transferrin is not a necessary condition for the presence of NTBI (9). The presence of NTBI among subjects with the normal range of percent transferrin saturation may be explained by mechanisms related to disturbance of iron homeostasis (9,16). In this sense, it may be important to note that the relationship between percent transferrin saturation and NTBI differed by group, even though the percent transferrin saturation values were very similar among the three groups. Although the percent transferrin saturation values were not closely related to the NTBI values in the control group, the correlation between these two parameters became significant in known diabetes. These trends emphasize the fact that percent transferrin saturation itself may be not a critical condition for the presence of NTBI. We speculate that a more critical factor may be the presence of a trigger that can disturb iron homeostasis; in this study, metabolic disturbances due to diabetes may act as a trigger. When people do not have any condition that can disturb iron homeostasis, stored body iron in the normal range may be harmless. However, once people have any condition that can disturb iron homeostasis such as diabetes, stored body iron that is reflected in percent transferrin saturation may release NTBI with a dose-response relation.

At present, the clinical importance of slightly increased NTBI levels in diabetes is unclear. Excess NTBI in type 2 diabetes may cause free radical formation and enhanced lipid peroxidation, increasing the risk of development of diabetes complications. Supporting this speculation, one prospective study reported that labile plasma iron, a component of NTBI that is both redox-active and chelatable, predicted mortality after myocardial infarction in patients with type 2 diabetes (17). However, in other studies (18,19), NTBI itself was not correlated with oxidative stress markers in the serum of hemodialysis patients who received iron therapy. In this study, NTBI in known diabetes showed a nonsignificant inverse associa-

tion with A1C. However, considering the unstable nature of NTBI in vivo, a single measurement of NTBI may not be sufficiently sensitive to permit estimation of the true association. Interestingly, two randomized, controlled clinical studies (20,21) have shown that phlebotomy led to decreased blood A1C levels, changes in insulin secretion and insulin resistance, and less vascular dysfunction in patients with type 2 diabetes. As regular phlebotomy among patients with hemochromatosis led to a substantial decrease of NTBI (15), the decrease of NTBI through phlebotomy among diabetic patients may be one of possible mechanisms of the above findings (20,21). Interestingly, in this study, elevation of NTBI was observed among subjects with newly diagnosed diabetes, but CRP levels were not elevated in them. This does suggest that elevation of NTBI may happen at an earlier stage of diabetes than elevation of CRP.

There is little consensus on the true level of NTBI or on how it should be measured. Recently, the first international interlaboratory evaluation of NTBI from a common serum sample set showed considerable differences in NTBI values between laboratories using different methods (22). In this study, NTBI measurement was performed blindly with respect to group membership; thus, low assay reproducibility would tend to attenuate the true association between NTBI values and diabetes status. In this study, we do not have information on vitamin C because the primary purpose of the current study was to explore whether NTBI presents in patients with type 2 diabetes whose percent transferrin saturation is in the normal range. The link between vitamin C, NTBI, and CVD complications should be clarified in a prospective study. In this study, hemoglobin level was also associated with NTBI in diabetic patients, raising a question whether the presence of NTBI might be just a consequence of excessive erythrocyte breakdown or hemolysis in these subjects. However, in general, hemoglobin level is correlated with iron markers. In this study, the correlations of hemoglobin with percent transferrin saturation or ferritin were 0.44 and 0.37, respectively. Thus, the correlations between hemoglobin and NTBI among the patients with newly diagnosed or known diabetes may reflect contributions from hemoglobin and other iron carriers. Similar to percent transferrin saturation, even though the mean level of hemoglobin was very similar between control subjects and those with newly diagnosed diabetes, the correlation between hemoglobin and NTBI was quite different between two groups. In addition, if erythrocyte breakdown or hemolysis were the primary source of NTBI, absolute levels of NTBI would be much higher than the levels observed in this study.

In summary, this study demonstrated that NTBI was commonly present in type 2 diabetic patients. Prospective cohort studies are needed to clarify the clinical importance of slightly increased NTBI levels and to further investigate our general hypothesis that vitamin C supplementation of type 2 diabetes may be harmful because such supplementation increases the likelihood of oxidative stress caused by redox active iron.

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