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Non-transferrin-bound iron is associated with biomarkers of oxidative stress, inflammation and endothelial dysfunction in type 2 diabetes

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ABSTRACT

Aims: To investigate the association between circulating non-transferrin-bound iron [NTBI], and markers of oxidative stress, endothelial function and inflammation in subjects with type 2 diabetes and non-diabetic subjects with varying degrees of obesity.
Methods: Plasma NTBI was measured by HPLC, together with total iron, iron-binding capacity, transferrin saturation and soluble transferrin receptor, together with total and reduced ascorbate, malondialdehyde [MDA], E-selectin and high-sensitivity c-reactive protein [hs-CRP] in groups of 28 subjects with type 2 diabetes, 28 non-obese controls and 17 obese non-diabetic subjects.
Results: Levels of NTBI were higher than controls in the diabetes group, but the total serum iron levels were lower. MDA levels were higher than controls in both the diabetes and obese groups, and this was associated with higher levels of oxidised ascorbate. hs-CRP levels were higher in both the diabetes and obese groups, and E-selectin was significantly higher in the diabetes group. There were strong positive correlations between HbA1c levels and NTBI [$P < 0.01$], HbA1c and E-selectin [$P < 0.001$] and NTBI and E-selectin [$P < 0.02$] in the diabetes group.
Conclusion: These results support the hypothesis that iron-mediated oxidative stress may be a mechanism linking poor glycaemic control with vascular dysfunction in type 2 diabetes.

1. Introduction

Diabetes and obesity are two closely associated conditions both carrying a risk of cardiovascular disease (Hubert, Feinleib, McNamara, & Castelli, 1983; Kannel & McGee, 1979). Although many factors are thought to influence the predisposition to cardiovascular disease the underlying mechanisms remain controversial. There is evidence that iron dysregulation may play a significant role in the vascular complications of diabetes, and cardiovascular disease in non-diabetic subjects (Basuli, Stevens, Torti, & Torti, 2014; Vinchi et al., 2014). In general, most studies have shown evidence of excessive iron stores in subjects with diabetes, while obese subjects tend to be iron deficient using standard methods of assessment (Yanoff et al., 2007; Zafon, Lecube, & Simó, 2010). However, the most appropriate methods to measure iron status and its potential impact on CVD risk are currently unclear.

Body iron is known to be present in a number of forms (Waldvogel-Abramowski et al., 2014), not all of which are available to contribute to the development of atherosclerosis or endothelial dysfunction. Within the body iron pool, it is generally considered that iron which is not sequestered on transferrin or bound to other iron-binding proteins, referred to as non-transferrin-bound iron [NTBI] contains a proportion of redox active iron capable of inducing oxidative damage to cells and tissues (Brissot, Ropert, Le Lan, & Loréal, 2012). This is a relatively small component of total iron stores but is potentially highly reactive (Brissot et al., 2012). The exact nature of NTBI remains to be elucidated, although it is considered that iron complexed with citrate may represent the major form (Brissot et al., 2012). Under certain conditions iron-citrate complexes are redox active and may contribute to oxidative damage to tissues (Adam, Bounds, Kissner, & Koppenol, 2015). Standard biomarkers for iron status such as ferritin and soluble transferrin receptor concentrations do not necessarily provide an accurate monitor of NTBI levels. In some cases changes in NTBI levels do follow changes in the levels of the standard biomarkers (Goto et al., 2013), but in others this is not so (Porter et al., 2014). Such findings have led to the suggestion that NTBI should be included as a valid independent biomarker of the

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potential adverse effects of iron in disease states (Lee & Jacobs, 2004; Taher et al., 2009).

The aim of this study, therefore, was to test the hypothesis that NTBI is a biomarker associated with indices of oxidative stress, inflammation and vascular dysfunction in individuals with type 2 diabetes and also in non-diabetic subjects with varying levels of obesity.

2. Materials and methods

2.1. Subjects

The study was approved by the National Health Service research ethics committee, and participants gave written informed consent. The study was conducted in accordance with the principles of the Declaration of Helsinki and Committee on the Harmonisation of Good Clinical Practice (Dixon, 1999). The study population was comprised of 73 subjects all aged >40 years: 28 had type 2 diabetes, recruited from diabetes clinics, 28 were lean healthy control subjects recruited by advertisement, and 17 were obese non-diabetic subjects recruited from diabetes clinics; 17 were obese non-diabetic subjects recruited from the general population and weight management clinics. Based on conservative assumptions on mean and standard deviation for NTBI measurements from previously published data (Lee et al., 2006) we estimated that a sample size of 23 subjects per group would give at least 90% power to detect a significant difference in NTBI between the diabetic and comparator control groups at the 5% significance level (http://www.stat.ubc.ca/-rollin/stats/ssize/). In support of this calculation, Leoncini et al. (2008) reported significant differences in NTBI levels between controls and subjects with diabetes using 24 subjects per group. We therefore set a recruitment target of 30 subjects per group, which would allow for up to 20% drop-outs.

Patients with diabetes had been suffering from the condition for 1–22 years post-diagnosis (mean ± SD: 9.79 ± 5.24 years). Recruitment occurred between November 2012 and November 2013. All diabetic subjects were receiving treatment to improve insulin sensitivity. Most (23) received metformin while others were treated with related drugs e.g., ploglitazone. Some subjects (12) also received treatment to improve insulin secretion, while others (7) received insulin or insulin substitute therapy. Only two of the subjects were treated for other morbidities, one for heart disease and one for asthma/COPD.

Anthropometric indices were measured which included BMI, body fat percentage (Tanita TBF-300MA Body Composition analyser, Tanita UK) and waist-to-hip ratio in a single visit. Each subject supplied 3 × 3 ml blood samples using Vacutainers (Becton Dickinson, Plymouth, UK), one with lithium heparin as anticoagulant, one with EDTA as anticoagulant and one plain tube for serum. Blood samples in EDTA and lithium heparin tubes were centrifuged at 1,000×*g* for 5 min to provide plasma; the blood in the plain tubes was centrifuged after 30 min at room temperature, and then plasma and serum were stored at −80 °C prior to biochemical analysis. From the EDTA plasma 400 μl were removed and added to 400 μl of 10% metaphosphoric acid (MPA) containing 2 mM EDTA and then centrifuged at 20,000×*g* at 4 °C for 10 min. The supernatant was removed and stored at −80 °C prior to the measurement of ascorbate.

2.2. Materials

Light magnesium carbonate, disodium EDTA, metaphosphoric acid, acetonitrile, ethanol, KCl, KH_2PO_4, NaCl, Na_2HPO_4•$12H_2O$, $FeCl_3$•$6H_2O$, Tris(2-carboxyethyl) phosphine hydrochloride (TCEP), phosphoric acid, 1,2-dimethyl-3-hydroxy-4(1H)-pyridinone (DHP), nitrilotriacetic acid (NTA), butylated hydroxytoluene, thiobarbituric acid and all other laboratory reagents were obtained from Sigma-Aldrich (Poole, UK) or Fisher Scientific (Loughborough, UK). Amicon Ultra filter units (0.5 ml, 30 kDa cut off) were purchased form Merck-Millipore (Fisher Scientific Loughborough, UK). ELISA kits for the measurement of E-Selectin and hsCRP were obtained from Biorbyt, UK. The ELISA kit for the measurement of soluble transferrin receptor was obtained from Biovendor, UK.

2.3. Measurement of total iron and total iron-binding capacity

Total iron and total iron-binding capacity were assessed by a scaled down version of the methods recommended by the International Committee for Standardisation in Haematology as described previously (Collard, White, & Copplestone, 2014). 400 μl of extracellular phase, blanks and standards were added to 400 μl of protein precipitation solution (0.6 M TCA and 0.4 M thioglycolic acid in 1 M HCl). This was mixed thoroughly for 1 minute and incubated at 56 °C for 15 minutes in a water bath. The samples were cooled and centrifuged for 5 minutes at 1,000×*g* to provide an optically clear supernatant. 500 μl of this was added to 500 μl of ferene 0.5 mM {3-(2-pyridyl-5,6-bis-[2-5-furyl sulphonic acid]-1,2,4 triazine} in 1.5 M sodium acetate. This was incubated for 5 minutes before absorbance was measured at 593 nm in a spectrophotometer. Iron concentration was computed from the absorbance of standards included in each batch of samples.

To determine iron-binding capacity, 350 μl of plasma was added to 350 μl of iron saturating solution (100 μM FeCl in 5 mM HCl). This was mixed and allowed to stand at room temperature for 5 minutes. 35 mg of light magnesium carbonate was then added, and the mixture agitated for 30 minutes. The magnesium carbonate was then removed by two sequential centrifugation steps of 1,000 × g for 5 minutes. 400 μl of the resultant supernatant was removed for the measurement of iron as described above.

2.4. Measurement of non-transferrin-bound iron [NTBI]

NTBI was measured using a slight modification of the HPLC method of Kime, Gibson, Yong, Hider, and Powers (1996). Briefly, 300 μl of plasma was incubated with 30 μl of 0.8 M nitrilotriacetic acid [NTA] for 20 minutes at room temperature to chelate loosely bound iron, such as that chelated with residual citrate or albumin. The samples were then placed in 30 kDa Amicon Ultra 0.5 ml filters (Millipore) and centrifuged at 13,000×*g* at 4 °C for 30 minutes. 250 μl of the ultrafiltrate were removed and incubated with 25 μl of 35 mM 3-hydroxy-1-propyl-2-methyl-pyridon-4-one for 5 minutes before injecting into the HPLC system (sample loop 20 μl). The mobile phase consisted of 5 mM PIPES buffer pH7.0 containing 3.5 mM 3-hydroxy-1-propyl-2-methyl-pyridon-4-one and 5% acetonitrile. The column was a PEEK lined 100 mm × 5 mm C18 column (Hichrom). All tubing was PEEK. The mobile phase was pumped at a flow rate of 1 ml/minute using a Dionex pump. The absorbance of the iron-chromophore complex was determined using a Dionex UV/VIS detector at a wavelength of 450 nm, and chromatography conducted using Chromeleon software. The concentration of NTBI was computed from blanks and standards taken through the whole procedure with each batch of samples. The coefficient of variation calculated using a range of NTBI concentrations over a number of days ranged from 3.79% to 7.50%.

2.5. Measurement of ascorbate

Total and oxidised ascorbate were measured by the method of Sato et al. (2010). Two aliquots of 90 μl were removed from the initial MPA extract. To one of these 10 μl of 5% MPA were added. A further 200 μl of 5% MPA was added, the sample mixed and injected into the HPLC system (20 μl sample loop). This provided the value for reduced ascorbate. To the other aliquot, 10 μl of tris (2-carboxyethyl) phosphine hydrochloride [TCEP] was added, and the mixture incubated at room temperature for 20 minutes to convert all the oxidised ascorbate to the reduced form. 200 μl of 5% MPA was then added, the sample mixed and injected into the HPLC system. This provided a measure of total