Free Radical Biology & Medicine 45 (2008) 1302–1307

Contents lists available at ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Ascorbyl free radical reflects catalytically active iron after intravenous iron saccharate injection

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ARTICLE INFO

Article history:
Received 6 December 2007
Revised 23 July 2008
Accepted 2 August 2008
Available online 12 August 2008

Keywords:
Ascorbyl free radical
Electron spin resonance
Hemodialysis
Iron saccharate
Non-transferrin-bound iron
Oxidative stress
Free radicals

ABSTRACT

Iron release from intravenous iron formulations can increase both non-transferrin-bound iron (NTBI) and oxidative stress. However, data showing a direct association between these parameters are sparse. The aim of this study was to adapt a recently published electron spin resonance (ESR) method to measure NTBI after iron injection and further to investigate its correlation to levels of oxidative stress markers. Twenty chronic hemodialysis patients were enrolled. NTBI and markers of oxidative stress, ascorbyl free radical (AFR), oxidized LDL, protein carbonyl, total antioxidant capacity, and myeloperoxidase, were measured in blood samples collected before and after intravenous injection of 100 mg iron saccharate. NTBI and all analyzed oxidative stress markers were increased 10 min after iron injection. Specifically, NTBI rose by 375% and AFR by 40%. Significant increases in these parameters were still seen 60 min after the injection. The changes in NTBI and AFR were closely correlated. The close correlation between intravascular release of NTBI and increase in plasma AFR after iv iron injection, as well as the increase in all measured oxidative stress markers, suggests that the iron measured was catalytically active. The ESR method was sufficiently sensitive and robust to measure NTBI also in human plasma.

Anemia is common in patients with chronic renal failure, due both to erythropoietin deficiency and to absolute or functional iron deficiency. According to universal guidelines, these patients are routinely treated with injections of ferric (Fe^{3+}) complexes as a supplement to treatment with recombinant human erythropoietin, resulting in improved hemoglobin values and reduced erythropoietin cost [1]. Intravenous iron injection has become the standard iron administration route, as per os supplementation is inadequate to maintain optimal iron stores in patients with end-stage renal disease [2,3]. Even though this route is not the natural way for iron to enter the body, iron injections have been shown to be clinically safe with relative few side effects. Nevertheless, there is a potential risk of getting undesired redox reactions due to the appearance of unbound iron in the circulation and, indeed, there are several reports that support this [4–6]. Unbound ferric iron is potentially hazardous to the body as it could rapidly be reduced to its ferrous state (Fe^{2+}) by any bioreductants available, such as ascorbic acid. Ferrous iron is toxic and can catalyze reactions associated with oxidative tissue damage. For example, it can mediate the formation of the noxious hydroxyl radical ($HO^{\cdot}$) via the Fenton reaction:

$$Fe^{2+} H_2O_2 \rightarrow Fe^{3+} + HO^{\cdot} + HO^{-}$$

Cardiovascular disease is the major cause of mortality and morbidity in patients with end-stage renal disease [7]. The pathogenesis is not entirely understood, but one explanation is the chronic low-grade systemic inflammation and increased oxidative stress found in this population [8–10]. As outlined above, parenteral iron treatment could induce oxidative stress, which in turn can lead to lipid peroxidation and atherosclerosis. In fact, clinical studies have indicated that increasing the total iron load through parenteral iron administration can induce oxidative stress and endothelial dysfunction, accelerate atherosclerosis, and speed up thrombus formation after arterial injury [11–22]. Further, hemodialysis patients with clinically evident congestive heart failure or ischemic heart disease did surprisingly worse when the hematocrit value was normalized with erythropoietin, possibly because of higher iron loading needed in the high hematocrit group [23]. Finally, in a retrospective study, hemodialysis patients with serum ferritin >600 μg/L had higher mortality compared to those with ferritin <600 μg/L, independent of the C-reactive protein level [24]. On the other hand, even though there is good evidence that non-transferrin-bound iron (NTBI)[1] appears after intravenous iron injection, the clinical relevance of this finding is still controversial. While there are several studies showing a significant correlation between the appearance of NTBI and oxidative

Abbreviations: AFR, ascorbyl free radical; NTBI, non-transferrin-bound iron; TIBC, total iron-binding capacity; ESR, electron spin resonance; hCRP, high sensitivity C-reactive protein; BDC-LDL, basal diene-conjugated low-density lipoprotein; DFO, desferrioxamine; TEAC, Trolox equivalent antioxidant capacity; MPO, myeloperoxidase; ESRD, end-stage renal disease; IS, iron saccharate; ID, iron dextran.

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doi:10.1016/j.freeradbiomed.2008.08.006

stress, peroxide generation, or endothelial dysfunction, others have not seen this connection [25–27].

The aim of this work was to adapt a previously published ESR method [28] to measure the level of NTBI after intravenously administrated iron saccharate in a population of chronic hemodialysis patients. Further, we wanted to evaluate if the measured iron is catalytically active by concurrently measuring various oxidative stress markers, such as ascorbyl free radical (AFR), which is easily measured by ESR.

Materials and methods

Patients

Twenty patients on chronic, regular hemodialysis treatment at the Hemodialysis Unit, Skaraborg Hospital, Skövde, Sweden, were enrolled in the study. All patients were clinically stable without any symptoms or signs of acute inflammation, infection, or heart disease. All patients had renal anemia, treated with subcutaneous erythropoietin injections and intravenous iron saccharate at a dose of 100 mg iv per week. The weekly dose of iron was unchanged for at least 3 months before the study (mean 9.7 ± 5.9 SD). No changes in iron regimens were made, and the last iron injection was 7 days before the trial. All patients were on daily vitamin substitution with thiamine 50 mg, riboflavin 5 mg, pyridoxine 5 mg, nicotinamide 200 mg, and ascorbic acid 100 mg. Main demographic, clinical characteristics, and biochemical analysis of the patients are summarized in Tables 1 and 2. The study protocol was approved by the local ethics committee and informed consent was obtained from all subjects.

Iron administration and blood sample collection

Experiment 1 was performed during the last 30 min of an ordinary dialysis session. Ferrous iron saccharate (100 mg, Venofer, Ferrum; Vifor, Inc., St. Gallen, Switzerland) was given as a single bolus injection into the venous chamber over 5 min. No adverse reactions were noted. Two blood samples were drawn from the infusion site of the arterial line, immediately before the iron injection and 10 min after the whole iron saccharate dose had been given. To evaluate further the effect of iron administration over time, a second experiment was carried out. In this experiment, eight patients were investigated further for 1 h after a regular dialysis treatment. Immediately after the dialysis session a blood sample was drawn from an access cannula remaining in the arteriovenous fistula, followed by injection of 100 mg iron saccharate over 5 min. Blood was then collected from the same cannula exactly 10, 30, and 60 min after the total iron dose had been given.

Table 1
Main clinical characteristics and dialysis modalities of the study population

Parameter	Value
Number of patients (*n*)	20
Mean age years (min/max)	59.9 (32/83)
Gender (male/female)	11/9
Diabetic nephropathy (*n*)	8
Chronic glomerulonephritis (*n*)	4
Nephrosclerosis (*n*)	3
Adult polycystic kidney disease (*n*)	2
Unspecified (*n*)	2
Chronic interstitial nephritis (*n*)	1
Proved cardiovascular disease (*n*)	12
Smokers (*n*)	2
Time with ESRD (months ± SD)	60.9 ± 88.8
Time on current HD treatment (months ± SD)	24.2 ± 19.6
Dialysis time per week (h ± SD)	12.04 ± 1.34
Dry weight (kg ± SD)	73.4 ± 25.7
High permeable membrane (*n*)	13
Kt/V	1.47 ± 0.19

Table 2
Mean biochemical values of the study population before ordinary hemodialysis treatment

Parameter	Value (mean ± SD)
Hemoglobin (g/L)	123.1 ± 13.7
s-Albumin (g/L)	36.1 ± 3.7
s-Iron (μmol/L)	10.5 ± 6.0
p-NTBI (μmol/L)	1.6 ± 0.3
s-TIBC (μmol/L)	43.5 ± 9.8
s-Transferrin saturation (%)	24.0 ± 12.7
s-Ferritin (μg/L)	304 ± 140
s-hCRP (mg/L)	6.6 ± 5.6

Blood sample preparation

Venous blood for various clinical chemical analyses was collected in serum Vacutainer tubes with clot activator. The blood for analysis of BDC-LDL was collected in EDTA Vacutainers, and blood for analysis of iron, total antioxidant capacity, NTBI, and AFR was collected in heparinized Vacutainers. Directly after collection, all samples were centrifuged at 3000 g for 10 min and then the plasma/serum was immediately stored at −80 °C until analysis.

Ascorbyl free radical analysis

The measurement of plasma AFR was made by electron spin resonance. Immediately after thawing of the sample, 50 μl of plasma was drawn into a capillary tube (i.d. 1 mm; KEBO-Lab, Stockholm, Sweden), which was sealed with hematocrit sealing compound (Brand GmbH, Wertheim, Germany). The AFR signal intensity was then measured with an X-band spectrometer Bruker ECS 106 (Bruker Biospin, Rheinstetten, Germany). All recordings were performed at room temperature. The instrument settings were microwave power, 10 mW; center field, 3480.30 G; sweep width, 5.0 G; modulation amplitude, 1.0 G; modulation frequency, 100.0 kHz; sweep time, 5.24 s; time constant, 163.84 ms; conversion time, 5.12 ms; and number of scans, 20. The AFR gives a characteristic two-peak signal and the mean height of the peaks was calculated to estimate the AFR concentration in the sample. The AFR concentration was determined using the stable nitroxide radical 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (Tempol) (Sigma–Aldrich, H 8258), 1 μM, as standard.

Measurement of non-transferrin-bound iron

NTBI was measured essentially as described by Kozlov et al. [28]. Plasma from hemodialysis patients and from healthy controls (450 μl) was mixed with 50 μl of 100 mM desferrioxamine (DFO) (Desferal; Novartis) and incubated for 15 min in room temperature. (Within this time frame any ferrous iron captured by DFO autoxidizes into ESR-detectable ferric iron, allowing detection of both ferric and ferrous iron chelated by DFO.) The samples were then filtered through Microcon YM 50 filters (Amicon/Millipore) with 50-kDa cutoff to eliminate possible interference from iron-containing proteins during ESR measurements. The filtered samples (250 μl) were transferred to quartz ESR tubes (Bruker E221004, i.d. 3 mm) and carefully frozen in liquid nitrogen. ESR spectra of the frozen samples were recorded at 150 K using an X-band ESR spectrometer (ELEXSYS; Bruker Biospin, Rheinstetten, Germany) equipped with a Dewar insert and a low-temperature system. The instrument settings were as follows: center field, 1632 G; sweep width, 200 G; microwave attenuation, 12 dB; modulation amplitude, 20 G; modulation frequency, 100 kHz; receiver gain, 60 dB; sweep time, 21 s; time constant, 164 ms; conversion time, 20 ms; resolution, 1024 points; number of scans, 10. The software package supplied with the ESR instrument (XEPR) was used to subtract the blank signal, obtained from 10 mM DFO in distilled water, from each sample spectrum. (The signal from 10 mM DFO, either in