Increase in plasma esterified F₂-isoprostanes following intravenous iron infusion in patients on hemodialysis

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Background. In epoetin-treated dialysis patients, currently iron is administered by the intravenous route to maintain optimum erythropoiesis. However, rapid infusion of iron in excess of transferrin binding capacity can lead to the availability of unbound iron that can theoretically catalyze peroxidation of lipids, such as low-density lipoprotein (LDL), which when oxidatively modified is proinflammatory and promotes atherogenesis.

Methods. To address this issue, our study used one of the most specific measures of lipid peroxidation available, namely gas chromatography/mass spectrometry (GC/MS) analysis of F₂-isoprostanes. Using a prospective design, blood samples were collected 15 minutes before (pre) and 30 minutes after (post) a one-hour infusion of 700 mg bolus of intravenous iron in 22 adult home-hemodialysis patients on a non-hemodialysis day.

Results. With iron-dextran infusion, serum iron markedly increased (mean ± SE, 42 ± 4 vs. 311 ± 92 µg/dL, P < 0.0001) and exceeded the transferrin saturation of 100% in 22 out of 22 patients (pre 23 ± 3 vs. post 165 ± 8%, P < 0.0001). Plasma concentrations of free F₂-isoprostanes did not change significantly following infusion of iron (pre 40 ± 5 vs. post 39 ± 6 pg/mL). However, levels of F₂-isoprostanes esterified in plasma lipoproteins increased significantly in the postinfusion samples (pre 199 ± 19 vs. post 233 ± 25 pg/mL, P < 0.004). Pre-infusion levels of serum iron correlated directly with pre-infusion levels of esterified F₂-isoprostanes (r = 0.56, P = 0.008), which persisted in the postinfusion period (r = 0.43, P = 0.04). However, there was no correlation between esterified F₂-isoprostanes and serum ferritin levels. In the last four patients in whom blood samples were collected five hours after the intravenous iron infusion, there were further increases in esterified F₂-isoprostanes that very closely correlated with postinfusion serum iron levels (r = 0.99, P = 0.013). In a control study, the in vitro addition of iron dextran to blood samples did not increase free or esterified F₂-isoprostanes, suggesting that the increase in esterified F₂-isoprostanes seen in vivo after iron infusion in patients is not due to a procedural artifact.

Conclusion. Collectively our data suggest that high levels of serum iron appearing soon after a large bolus of iron infusion is associated with significant, albeit modest, increases in levels of F₂-isoprostanes esterified in plasma lipoproteins that tended to increase with time. Although it is uncertain whether this degree of lipid peroxidation may have deleterious effects, it may be sagacious to explore whether this can be prevented by slow infusion of frequent smaller doses of iron and, if necessary, along with administration of antioxidants.

Iron infused intravenously in large doses may exceed the binding capacity of transferrin and lead to the availability of unbound iron in the blood [1, 2]. Unbound iron can catalyze by the Haber-Weiss reaction, thus generating highly reactive hydroxyl radicals that promote lipid peroxidation. A prevailing and experimentally supported mechanism for atherogenesis is based on free radical-catalyzed oxidation of lipoproteins. Oxidized low-density lipoprotein (LDL) is proinflammatory and has been shown to promote atherogenesis. Macrophages/monocytes scavenge oxidized LDL in the subendothelial space, which results in foam cell formation that then elaborates pro-inflammatory cytokine and growth factors, and up-regulates adhesion molecules [3, 4]. The leading cause of mortality in the dialysis population is cardiovascular disease related to coronary atherosclerosis [5]. In theory, repeated intravenous iron infusion can promote LDL peroxidation and, through the aforementioned mechanism, may accelerate atherosclerosis in dialysis population. Potentially this may worsen the already elevated mortality rate in the dialysis population and undermine the benefits of improved anemia correction from repeated intravenous iron infusion. The suggestive evidence that increased intravenous iron administration may increase the risk for cardiac events comes from Besarab et al’s study in which higher cardiac events and mortality were observed in the normal hematocrit (Hct) group who, among other things, also received more intravenous iron [6]. Although the difference in mortality and cardiac events between the two groups did not reach statistical significance, the study was halted [6].

Recent studies using plasma malondialdehyde (MDA) measured by the thiobarbituric acid-reacting substances...
(TBA) test and plasma peroxides suggested that intravenous iron infusion is associated with marked lipid peroxidation [2]. However, these methods are nonspecific and are subject to false positive results. Measurements of peroxides can be used in studies on purified lipids, but its use in the biological system is questionable due to interference from many other oxidized species present in plasma [7]. Similarly, lipid peroxidation determination based on the TBA test, which involves heating the testing material with thiobarbituric acid and measuring the pink color formed in the process at 532 nm, can provide spurious positive results [7]. The lack of specificity of the TBA assay when applied to human plasma is amply demonstrated by the work of Warso and Lands and is discussed in detail by Halliwell and Gutteridge [8, 9]. Use of iron by an intravenous route has turned out to be a boon for the management of anemia in dialysis patients as it is simple, efficacious, and generally safe. Thus, it is critical to verify whether there is a true occurrence of lipid peroxidation with iron infusion and, if so, whether there is peroxidation of lipoproteins, an event considered critical in atherogenesis. We addressed this issue using one of the most specific measures of lipid peroxidation currently available, namely gas chromatography/mass spectrometry (GC/MS) measurement of F2-isoprostanes [10]. These are formed in vivo by the free radical-induced peroxidation of arachidonic acid, independent of the cyclooxygenase enzyme [11]. Unlike the cyclooxygenase-catalyzed prostaglandin production, F2-isoprostanes are initially formed in situ esterified to tissue lipids and circulating lipoproteins, and subsequently are released in free form [12, 13]. A large body of evidence in experimental animals and humans indicates that measurement of F2-isoprostanes represent one of the most reliable approaches to assess lipid peroxidation including LDL peroxidation and oxidant stress in vivo [10, 14, 15].

To test whether intravenous iron infusion is associated with an increase in free and esterified plasma F2-isoprostanes, plasma samples were prospectively collected just before and one half hour after the one-hour infusion (700 mg) of iron dextran in home hemodialysis patients on a non-hemodialysis day. The results demonstrate that there was a modest increase in F2-isoprostanes esterified on plasma lipoproteins that correlated with serum iron concentration, suggestive of the occurrence of iron-induced lipid peroxidation.

**METHODS**

The study protocol, which was approved by the Institutional Review Board of University of Mississippi Medical Center, was carried out prospectively in 22 patients on home hemodialysis who were managed by the Renal Care Group Inc. of Jackson (MS, USA). The selection criteria were that patients should be between 20 to 75 years old, had to be clinically stable, established on home hemodialysis for at least six months, and scheduled for intravenous iron administration based on clinical indication. No change was made in the routine intravenous iron-infusion protocol for the study except that blood samples were collected before and after iron infusion. Prior to the study, the patients’ demographics, medication and dialysis details, and clinical conditions were assessed. Also obtained was the dose of total iron infused in each of the study subjects during the preceding 12 months.

The dose of iron dextran administered to our patients on home-hemodialysis was 700 mg and was ordered by the patient’s nephrologist based on a “need-based” indication for iron repletion. The iron-infusion studies in all 22 patients were carried out by the same research nurse (BO), and the iron dextran preparation used in this study was from Schein Pharmaceutical Inc. (Florham Park, NJ, USA). Iron infusion was carried out on a non-dialysis day to avoid any confounding effect of hemodialysis on the study. Pre-iron-infusion samples were collected 15 minutes prior to administration of iron including the test dose. A 21-gauge plastic catheter was inserted in the patient’s hemodialysis vascular access to draw blood samples. A needleless system with a Leur-lock adapter was attached to the catheter and flushed with 2 mL 0.9% saline. A 1 mL waste was drawn prior to all collections to eliminate the possibility of diluting the samples by the saline. All iron dextran, including the test dose, was administered via a peripheral infusion site in the extremity opposite the blood-sampling site. All samples were drawn into a prechilled syringe and transferred to a prechilled transfer tube. A 5 mL tube with potassium ethylenediaminetetra-acetic acid (EDTA) was used for the F2-isoprostanes samples, and a 10 mL SST Gel and clot activator tube was used to collect the other iron-related measurements. Samples for F2-isoprostanes were immediately centrifuged, and plasma was transferred to a prechilled transfer tube and submerged in dry ice until transport to the −84°C freezer for storage. All infusions were timed to run for one hour. The postinfusion samples were drawn 30 minutes after the completion of iron infusion. In last four patients, an additional sample was drawn at five hours postinfusion (late post) to determine whether there was a time-related increase in plasma F2-isoprostanes after iron infusion. A control study was also undertaken to determine whether processing of the sample for F2-isoprostanes could lead to ex vivo F2-isoprostane formation. For this study, iron dextran was added to blood samples from healthy volunteers to obtain similar iron levels as in patients after iron infusion. Samples were then processed in the same fashion as the patients’ samples and were subjected to F2-isoprostane measurements.

Samples were packed in dry ice during shipment to Vanderbilt University for the F2-isoprostane measurement, and all samples were verified to have arrived in a
was associated with a significant increase in serum iron levels either before or after iron infusion. The BP did not change significantly with an increase in hematocrit. Plasma-esterified isoprostanes were measured five hours after the completion of iron infusion as transferrin saturation in 22 out of 22 patients exceeded 100% (serum iron/TIBC × 100) exceeded 100% in 22 patients (pre 23 ± 3 vs. post 165 ± 8%, P < 0.0001), and this was associated with a significant increase in serum iron (42 ± 4 vs. 311 ± 92 μg/dL, P < 0.0001). Serum transferrin and ferritin concentrations did not change.

Effect of intravenous iron administration on free and esterified F₂-isoprostanes

Plasma concentrations of free isoprostanes did not change significantly following infusion of iron (pre 40 ± 5 vs. post 39 ± 6 pg/mL). However, levels of isoprostanes esterified in plasma lipoproteins increased significantly in the postinfusion samples (pre 199 ± 19 vs. post 233 ± 25 pg/mL, P < 0.004; Fig. 1A). The levels rose in 16 out of 22 patients, fell in four, and were unchanged in two (Fig. 1B).

In the last four patients in whom a five-hour post infusion sample was collected (late post), the free isoprostanes did not increase. The pre-, post-, and late-post values were 33 ± 6, 29 ± 4, and 28 ± 2 pg/mL, respectively. However, the mean esterified isoprostanes did increase with time insignificantly (N = 4), and the respective levels were 332 ± 39, 379 ± 60, and 398 ± 46 pg/mL. The comparison between pre-iron and post-iron infusion data analysis. The results are reported as mean ± SEM. The correlation was assessed by simple linear regression. P < 0.05 was considered statistically significant.

Relationship between esterified isoprostanes and serum iron and iron-related parameters

There was a significantly positive correlation between levels of serum iron and esterified isoprostanes prior to iron infusion (r = 0.56, P = 0.008; Fig. 2A). Following iron infusion, serum iron levels rose rapidly and the relationship between iron levels and esterified isoprostanes, while persistent, became weak soon after iron infusion (r = 0.44, P = 0.042; Fig. 2B). In four patients in whom plasma-esterified isoprostanes were measured five hours after the completion of iron infusion, there was a remarkably close correlation between the five-hour post infusion levels of iron and esterified isoprostanes (r = 0.99, P = 0.013; Fig. 2C). Transferrin saturation showed a positive correlation with the pre-iron infusion levels of esterified isoprostanes (r = 0.49, P = 0.022), but this was lost after iron infusion as transferrin saturation in 22 out of 22 exceeded 100% (r = 0.10, P = 0.68). There was no correlation between plasma isoprostanes and serum ferritin levels either before or after iron infusion.

| Table 1. Serum iron and iron-related parameters in samples taken 15 minutes before and one-half hour after the one-hour infusion of 700 mg of iron dextran |
|---------------------------------|--------|--------|----------|
| Iron infusion                   | Pre    | Post   | P value  |
| Serum iron μg/dL               | 42 ± 16| 311 ± 92| <0.0001 |
| Serum ferritin ng/mL           | 348 ± 74| 359 ± 71| NS |
| TIBC                           | 183 ± 46| 191 ± 49| NS |
| Serum transferrin mg/dL        | 144 ± 37| 151 ± 39| NS |
| TSAT %                         | 22 ± 7 | 165 ± 36| <0.0001 |

Data are mean ± SEM. Abbreviations are: TIBC, total iron-binding capacity; TSAT, transferrin saturation.
iron dextran infusion that appear to exceed the binding capacity of transferrin were not immediately associated with generalized lipid peroxidation. However, the findings of a modest but significant increase in plasma esterified F₂-isoprostanes after iron infusion and the presence of a strong correlation between esterified isoprostanes and serum iron concentrations suggest that iron infusion may cause low-grade and ongoing peroxidation of lipids in the plasma, which are contained in lipoproteins.

Iron and other redox metal ions are strong catalysts for LDL oxidation and, as alluded to earlier, the latter is widely regarded as a seminal process in the pathogenesis of atherosclerosis [3, 18–20]. Sullivan hypothesized a link between body iron and atherosclerosis to explain the gender difference in the incidence of heart disease [21], and some, but not all, studies support an association between high levels of iron and the occurrence of heart disease [22–27]. Employing a more specific measurement of body iron stores such as the ratio of serum transferrin receptor to serum ferritin, a recent prospective study showed an association between increased body iron stores and an excess risk for myocardial infarction in the general population [22, 28]. Data in our study demonstrate a strong correlation ($r = 0.56$) between the concentrations of serum iron and plasma-esterified F₂-isoprostanes, suggesting that higher plasma iron concentrations lead to enhanced lipoprotein peroxidation. For these measurements in our study, technologists blinded for sample details measured the samples and each of these parameters.

![Graph A](image1.png)

**Fig. 1.** (A) Levels of esterified F₂-isoprostanes in the plasma obtained 15 minutes before (pre) and 30 minutes after (post) the one-hour infusion of 700 mg of iron dextran in 22 patients on a non-hemodialysis day. Data are mean ± SEM; *P < 0.004 pre-esterified vs. post-esterified F₂-isoprostanes. (B) The pre-iron and post-iron infusion levels of esterified F₂-isoprostanes in individual patients.

**Effect of in vitro addition of iron dextran to blood on isoprostane levels**

To determine whether the increase in esterified isoprostanes in patients after intravenous iron infusion could be a procedural artifact, that is, ex vivo autoxidation, iron dextran was added to blood samples from five healthy volunteers (3 males, age 36 ± 5 years, serum creatinine 0.9 ± 0.1 mg/dL) such that the serum iron levels rose from 105 ± 11 to 336 ± 35 μg/dL, P < 0.0001. No differences were seen in the levels of F₂-isoprostanes in plasma to which iron had been added compared to levels in plasma to which no iron had been added. Free isoprostanes before and after added iron were 71.0 ± 9.5 and 71.7 ± 9.5 pg/mL, and the respective values for esterified isoprostanes were 287.8 ± 34.5 and 280.6 ± 33.5 pg/mL.

**DISCUSSION**

Our study, based on the measurement of free plasma F₂-isoprostane levels, demonstrates that large doses of iron dextran infusion that appear to exceed the binding capacity of transferrin were not immediately associated with generalized lipid peroxidation. However, the findings of a modest but significant increase in plasma esterified F₂-isoprostanes after iron infusion and the presence of a strong correlation between esterified isoprostanes and serum iron concentrations suggest that iron infusion may cause low-grade and ongoing peroxidation of lipids in the plasma, which are contained in lipoproteins.
exposed to a higher concentration of iron during intravenous infusion than lipids in peripheral tissues. The marked increase in lipid peroxidation noted in one previous study could be due, at least in part, to the occurrence of spurious lipid peroxidation in vitro [2]. While employment of high-pressure liquid chromatography (HPLC) achieves greater specificity in separating the TBA-MDA adduct from the reaction mixture, it must be noted that exposure of carbohydrates and amino acids to hydroxyl radicals yields products that give a genuine TBA-MDA adduct in the TBA assay [9]. Thus, TBA assay used in the previous study is not specific for lipid peroxidation [2]. An additional issue with the TBA method is that MDA can be formed in vitro during the assay procedure, and its application to human body fluids also will measure endoperoxides produced enzymically by the prostaglandin synthesis pathway [9]. Moreover, platelets are activated and generate thromboxane during sampling of blood, and the thromboxane synthase generates one molecule of MDA for each molecule of thromboxane formed [29]. For these considerations, we addressed this issue using one of the most specific measures of lipid peroxidation available, namely GC/MS analysis of F2-isoprostanes [10, 14, 15].

Iron administration by the intravenous route is the most effective and convenient way to administer iron in dialysis patients receiving erythropoietin. In recent studies that employed iron administration by the intravenous route, there was a significant reduction in the erythropoietin dose while maintaining target hemoglobin levels. While employment of high-pressure liquid chromatography (HPLC) achieves greater specificity in separating the TBA-MDA adduct from the reaction mixture, it must be noted that exposure of carbohydrates and amino acids to hydroxyl radicals yields products that give a genuine TBA-MDA adduct in the TBA assay [9]. Thus, TBA assay used in the previous study is not specific for lipid peroxidation [2]. An additional issue with the TBA method is that MDA can be formed in vitro during the assay procedure, and its application to human body fluids also will measure endoperoxides produced enzymically by the prostaglandin synthesis pathway [9]. Moreover, platelets are activated and generate thromboxane during sampling of blood, and the thromboxane synthase generates one molecule of MDA for each molecule of thromboxane formed [29]. For these considerations, we addressed this issue using one of the most specific measures of lipid peroxidation available, namely GC/MS analysis of F2-isoprostanes [10, 14, 15].
studies. Whether an increase in this form of body iron may lead to toxicity is unlikely, because the iron in ferritin is in the storage form and is not readily bioavailable. Consistently, ferritin levels did not influence F₂-isoprostane levels in this study. Furthermore, that the high level of ferritin per se may not directly contribute to iron toxicity is supported also by the observation of the lack of undue atherosclerosis in patients and models of hemochromatosis [26, 35]. Unlike ferritin, our study suggests that the high levels of serum iron that often accompany high TSAT might become available for catalyzing lipid peroxidation. Thus, the concern ought to be about the toxicity of high peak concentrations of iron in the blood, which occurs during and days immediately following the intravenous bolus of large quantities of iron [31]. Smaller doses of iron administered over the duration of each dialysis can avoid the high peak and trough levels of iron. This will avoid potentially overloading the binding capacity of transferrin. If iron was administered at a lower dose (that is, 12.5 to 25 mg) and more frequently (that is, each dialysis over 4 hours), the bioavailable iron could be expected to be present persistently without the risk for high serum iron levels and transferrin supersaturation. Thus, administering lower doses of iron more frequently, at least in theory, is unlikely to lead to plasma lipoprotein peroxidation and tissue iron deposition.

Although our patients were on home hemodialysis, to receive the intravenous iron dextran infusion under medical supervision, they attended a dialysis center. These patients were not on dialysis on the day of infusion. The clinical routine was that a “total dose” of 700 mg iron dextran was to be infused once a month until iron stores were replete. Our finding that iron dextran infusion in large doses can exceed the transferring binding and lead to modest lipid peroxidation suggests that unbound iron as theorized could be a catalyst for lipid peroxidation. In childhood iron poisoning, serum iron concentrations over 250 μg/dL can be high enough to exceed the total number of transferrin binding sites in serum [36]. However, it should be noted that certain types of serum assays for iron measure the iron present in iron complexes spuriously. This is particularly true for ferric gluconate rather than the iron dextran used in our study, and can lead to erroneously high results for the oversaturation of transferrin [16]. The iron assay based on the Ferrozine method employed in our study also is reported to be more specific in that in an in vitro study, the Ferrozine method measured only 2.2% (±1.2%) of the iron present in iron dextran when iron dextran was added to serum [16].

It is possible that at a lower dose of 100 mg of iron dextran typically administered in patients attending limited-care dialysis facilities, the TSAT may not exceed 100% and unbound iron-mediated oxidative stress and lipid peroxidation may not occur. On the other hand, Roob et al reported the occurrence of lipid peroxidation even with 100 mg of iron sucrose infusion [2]. In that study, however, iron was infused while the patients were undergoing hemodialysis. Hemodialysis might in itself provoke oxidative stress through a number of mechanisms [37, 38] and, therefore, it is possible that ongoing hemodialysis procedure might set the stage for a greater oxidative stress even at a lower dose of iron infusion. Studies using more specific measures of oxidative stress such as F₂-isoprostane measurements by mass spectroscopy are required to verify whether iron infusion at 100 mg per dose is devoid of any potential oxidative effect.

A significant increase in esterified F₂-isoprostanes reflects the increase in total plasma lipoprotein oxidation, considered mostly to be of LDL origin, as LDL form the bulk and are susceptible to oxidation [39, 40]. There were only four patients with diabetes mellitus in this study, and their pre-iron or post-iron infusion of esterified isoprostanes was not different from the rest of the study subjects. Our patients were routinely prescribed non-vitamin E-containing multivitamins, but none was on vitamin E. One patient took over-the-counter multivitamins. Thus, the reason for the modest increase noted in esterified isoprostanes in this study or lack of increase in esterified isoprostanes in four patients with iron infusion cannot readily be ascribed to variability due the effect of any significant antioxidant intake. However, studies are required to test whether antioxidants can attenuate iron-induced increase in plasma F₂-isoprostanes.

When this study was designed, we predicted that if there were to be lipid peroxidation after intravenous bolus of iron, it would likely be present within an hour or two of infusion. The finding that isoprostane levels increases at five hours after infusion suggested that lipid peroxidation after iron infusion is a slow and accruing phenomenon that might take several hours or possibly days to peak after iron infusion. Consistent with this notion, the strongest correlation between levels of serum iron and esterified isoprostanes was seen in the pre-infusion levels.

In summary, these data indicate that infusion of high doses of iron is associated with very high levels of serum iron, saturation of transferrin, and significant, albeit modest, increase in F₂-isoprostanes esterified in plasma lipoproteins. Although it is uncertain whether this degree of lipoprotein oxidation may have deleterious effects, it may be prudent to explore whether this can be prevented by modification of the protocol using slow infusion of smaller and more frequent doses of iron and, if necessary, that the infusion be along with antioxidant administration.

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