Lipid Peroxidation and Protein Modificationin a Mouse Model of Chronic Iron Overload

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Iron-storage diseases are believed to cause organ damage through generation of reactive oxygen species. Using a murine model of iron overload, we found that hepatic iron stores increased logarithmically during 3 weeks of chronic intraperitoneal administration of iron dextran, while hepatic glutathione peroxidase activity declined linearly by approximately 50% during the same period. Plasma concentrations of aliphatic aldehydes increased by 2- to 3-fold, and plasma malondialdehyde (MDA) by 6-fold. Modification of total liver protein by products of lipid peroxidation, including MDA-lysine, 4-hydroxynonenal-lysine, and N^{ϵ}-(carboxymethyl)lysine (CML), increased by approximately 3-fold, while levels of the protein oxidation marker, methionine sulfoxide (MetSO), were unchanged. Skin collagen was resistant to modification until the third week, when 2- to 3-fold increases in both CML and MetSO were observed. Our results document that iron overload increases lipid peroxidation, with concomitant increases in reactive aldehydes in plasma and chemical modification of tissue proteins. CML was a sensitive indicator of hepatocellular oxidative stress, compared to MetSO, while extensive modification of extracellular skin collagen was not observed until the late stages of iron overload and oxidative stress. These observations provide direct evidence for the contribution of reactive oxygen species, lipid peroxidation, and reactive carbonyl intermediates to the pathogenesis of iron-overload diseases.

IRON IS AN ESSENTIAL element for normal cell function and metabolism. However, in excess quantities secondary to iron-loading and released from proteins (eg, ferritin, transferrin), it is highly cytotoxic.[1,2] Iron-induced liver damage and failure are associated with both primary and secondary hemochromatosis, common genetic disorders of iron metabolism.[3,4] The liver is the primary storage site for iron, and elevated hepatic iron concentration has been linked to hepatomegaly, cirrhosis, and hepatocellular cancers.[5-7] The exact mechanism of iron-induced injury and organ failure remains to be elucidated, but iron's ultimate toxicity is thought to result from its catalytic role in free radical generation via Fenton-type reactions, leading to oxidative damage to all classes of biomolecules.[2,8,9]

There is evidence showing that excess hydroxyl radical generation occurs subsequent to iron-loading in rats, based on electron spin resonance trapping experiments .[10-13] One consequence of radical damage should be increased lipid peroxidation, since lipids are among the most readily oxidizable substrates in biological systems. Increased concentrations of aldehyde-derived lipid peroxidation products have been demonstrated in experimental models of iron overload[14-19] and in patients with hereditary hemochromatosis[20-22] or β-thalassemia major.[23-27] Further, patients with iron-overload disorders have significantly increased concentrations of potentially toxic and reactive non–protein-bound iron in plasma.[20,28-30] Nevertheless, the dose-dependent effects of iron-induced organ failure remain poorly understood[17,18] and the chemical modifications of biomolecules resulting from reactive carbonyl compounds have not been fully characterized and quantified. We hypothesized that in a murine model of chronic iron overload there would be a dose-dependent increase in radical generation, leading to increased oxidative damage to proteins, measured as formation of methionine sulfoxide (MetSO),[31] and formation of advanced lipoxidation endproducts on proteins, measured as malondialdehyde-lysine (MDA-Lys), 4-hydroxynonenal-lysine (HNE-Lys),[32] and N^{ϵ}-(carboxymethyl)lysine (CML).[33] To investigate these hypotheses, we assessed the effects of chronic iron-loading on plasma aldehyde content and chemical modification of liver protein and skin collagen by products of oxidation reactions. We also measured glutathione peroxidase (GPx) activity, a critical component of the hepatic antioxidant defense mechanism.

MATERIALS AND METHODS

Materials

Unless otherwise indicated, all chemicals and reagents were purchased from Sigma-Aldrich (St Louis, MO).

Animal Model, Iron Administration, and Tissue Sampling

This investigation had institutional approval and conformed to the standards of the Canadian Council on Animal Care.[34] Male B6D2F1 mice (Jackson Laboratory, Bar Harbor, ME), 3 to 5 weeks of age and weighing 20 to 25 g, were adapted to their surrounding for a period of

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3 days prior to experimentation. The mice were housed in stainless steel cages (5 per cage) in a temperature- and humidity-controlled room with 12-hour light-dark cycles. Rodent Diet 5001 (PMI Nutrition International, Inc, St Louis, MO) contained 184 ppm of iron; mice had access to food and water ad libitum

Mice were randomized to receive treatment with placebo or iron for various lengths of time, as detailed below. We have previously demonstrated that parenteral iron loading of B6D2F1 mice results in dose-dependent increases in the concentration of iron in major organs (heart, liver, spleen), including both hepatocytes and Kupffer cells in liver.[18,19,35] The animals exhibited classical signs of iron toxicity including coarse fur, lethargy, and abdominal ascites. As detailed elsewhere, this animal model is most pertinent as a model of β-thalassemia .[17-19,35]

Iron-loading was achieved by intraperitoneal (IP) injections of iron dextran (Imferon, Sigma, St Louis, MO) in 0.5 mL saline (20 mg/d per mouse), on 5 of 7 days per week, for 1 week (100 mg total iron dose, n = 15), 2 weeks (200 mg total iron dose, n = 15), or 3 weeks total (300 mg total iron dose, n = 15). Animals were killed 24 hours after the last iron dextran injection. Control mice (n = 15) received placebo treatment with normal saline IP (0.5 mL/d per mouse) for a total period of 3 weeks.

Following each treatment period, mice were anesthetized with a mixture of ketamine hydrochloride (90 mg/kg IP) and Rompun (xylazine, Haver-Lockhart; 10 mg/kg IP), and a blood sample (~0.5 mL) was obtained via cardiac puncture using a heparinized syringe. The blood was centrifuged immediately for 15 minutes at 3,200 rpm (1,090 × *g*) at 2°C in an Eppendorf centrifuge (model 5417R, Brinkmann Instruments, Westbury, NY). Plasma was removed and stored at −70°C until analyzed. After removing the blood sample, mice were killed by cervical dislocation, and the livers were removed and stored at −70°C until analyzed.

Total Iron Concentrations

Total iron concentration in representative livers (5 per treatment group) was assessed by flame atomic absorption on an IL-551 Atomic Absorption/Atomic Emission Spectrophotometer (Instrumentation Laboratory, Wilmington, MA), as described previously.[36] The detection limit of this procedure was 2 μg/L, and total iron concentrations were compared to in-house control material and National Institute of Standards and Technology (NIST; Inorganic Ventures, Lakewood, NJ) calibration standards.

GPx Activity

GPx activity in liver tissue (5 per treatment group) was quantified by scanning fluorescence spectrophotometry on a COBAS-FARA centrifugal analyzer (Varian Techron TT Ltd, Balgrave, Australia), as described elsewhere.[18,37] A commercially available standard (Randox Laboratories Ltd, Ardmore, Crumlin, UK) for GPx activity was used for control of accuracy and precision.

Analysis of Plasma Aldehydes by Gas Chromatography/Mass Spectrometry

Systemic free radical generation was quantified by the presence of 20 saturated and unsaturated aldehydes (C_2-C_{12}) in plasma, as detailed previously.[38,39] Briefly, this technique is based on the use of O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride to form a pentafluorobenzyl-oxime derivative (PFB-oxime), followed by trimethylsilylation of the hydroxyl group to trimethylsilyl (TMS) ethers. The PFB-oxime-TMS derivatives were analyzed on a VG (Manchester, UK) Trio 2A triple quadrupole mass spectrometer, using a Hewlett Packard (Sunnyvale, CA) 5890 Series II gas chromatograph equipped with a 30-m, 0.32-μm DB-5 capillary column. The detection limit of this method is 50 to 1,00 fmol per 1 μL of injected aldehyde.[38] Total plasma aldehydes were calculated by summing the values for the 20 individual aldehydes, measured for each mouse separately, and mean values were determined for each treatment group.

Protein Isolation

Liver protein was isolated according to the procedure of Pamplona et al.[40] Briefly, liver was homogenized at 4°C in a Potter-Elvejhem homogenizer in deionized water containing 5 mmol/L diethylenetriaminepentaacetic acid. An equal volume of methanol was added and the sample rehomogenized. The homogenate was transferred to a screw-cap test tube and extracted sequentially with chloroform and diethyl ether, then centrifuged to pellet-extracted protein. The protein was dried by centrifugal evaporation in a Savant Instruments (Farmingdale, NY) Speed-Vac and stored at –70°C until analyzed. Skin was scraped free of adventitious tissue using a razor blade, then sequentially extracted with salt, acetic acid, and organic solvent to isolate insoluble collagen, as described previously.[41]

Analysis of Protein Modification and Oxidation

CML, HNE-Lys, and MDA-Lys were quantified in liver protein and skin collagen using isotope dilution mass spectrometry, as previously described.[32] Briefly, following $NaBH_4$ reduction, liver proteins were precipitated with 10% trichloroacetic acid, then heavy-labeled internal standards (d_4-CML, d_8-HNE-Lys, d_8-MDA-Lys, d_8-lysine) were added. For skin collagen, following $NaBH_4$ reduction, samples were washed several times with deionized water before adding internal standards. All samples were hydrolyzed in 6N HCl at 110°C for 24 hours, dried, and then applied to a C_{18} Sep-Pak column (Waters, Milford, MA) and eluted with 0.1% trifluoroacetic acid in 20% methanol; brown impurities were retained on the column. Modified amino acids were converted to their *N*-trifluoroacetyl methyl ester (TFAME) derivatives, and analyzed by selected-ion monitoring (SIM)–gas chromatography/mass spectrometry (GC/MS). These analyses were performed on a Hewlett-Packard model 5890 gas chromatograph interfaced to a VG 70-SQ magnetic sector mass spectrometer.[32] The detection limits for this assay are 50 fmol per 1-μL injection for the various analytes. Quantities of the modified amino acids were expressed as a ratio to the parent amino acid lysine.

MetSO was analyzed in tissue protein in a separate hydrolysis using 3 mol/L methanesulfonic acid, to avoid HCl-catalyzed conversion of MetSO to methionine (Met).[42] Deuterated MetSO and Met were used as internal standards, and were added prior to acid hydrolysis. Following hydrolysis, amino acids were isolated by ion exchange chromatography, then analyzed by SIM-GC/MS as their trimethylsilyl derivatives[43]; the detection limit for this assay is 5 pmol per 1-μL injection.[44]

Statistical Analyses

The Statistical Analysis System (SAS Institute, Cary, NC) was the principal software package employed for data analysis. Descriptive statistics for the key variables are presented as the mean ± SD. To establish the relationship between the key variables, Pearson's product moment correlation analysis was performed. Data analysis for treatment comparison followed a 2-step procedure. First, 1-way analysis of variance (ANOVA) was conducted to compare overall treatment differences, and a *P* value less than .05 was deemed significant. When a statistically significant difference was detected, post-hoc multiple pairwise comparisons were performed using Duncan's multiple range test.

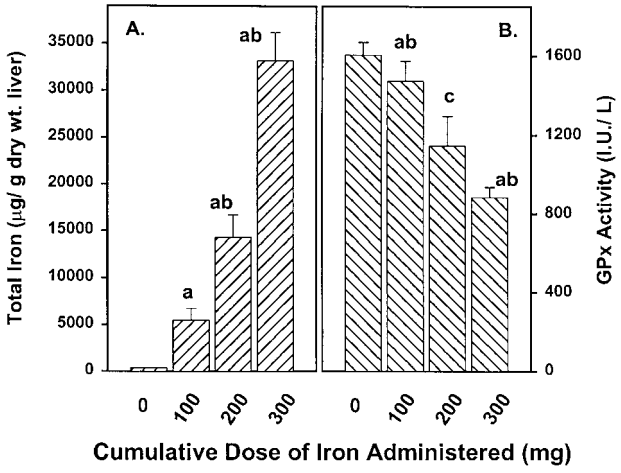

Fig 1. Total hepatic iron concentration (A) increases and GPx activity (B) decreases during chronic iron administration to mice. Livers were removed after parenteral administration of indicated amounts of iron. Total iron concentration and GPx activity were determined as described in the text. Data are mean ± SD (n = 5 per group). (A) $^{a}P < .001$ compared to control, and $^{b}P < .01$ compared to 100 mg iron. (B) $^{a}P < .001$ compared to control, $^{b}P < .01$ compared to 200 mg iron, and $^{c}P < .03$ compared to control.

RESULTS

Effect of Chronic Iron Administration on Body and Liver Weight, and on Liver Iron Content and GPx Activity

As expected from previous studies,[17-19,35,37] chronic administration of iron resulted in a progressive decrease in body weight, from a mean of 24 ± 1.1 g at week 0 to 16.5 ± 1.1 g by the time mice were killed at week 3. Over the time frame of the experiment, there was a progressive increase in liver weight from 1.4 ± 0.1 g to 2.4 ± 0.1 g, and the livers became bronze in color. Total liver iron content increased nearly exponentially with increasing dose of iron administered (Fig 1A). At the same time, the activity of the antioxidant enzyme GPx (Fig 1B) declined to about 50% of its basal value by 3 weeks. Although iron accumulation was exponential, GPx activity declined in a linear fashion.

Plasma Aldehyde Accumulation During Chronic Iron Overload

Table 1 summarizes the plasma concentrations of 20 individual aldehydes with increasing dose of iron. In comparison to untreated mice, there was a 2- to 3-fold dose-dependent increase in the concentration of the majority of aldehydes in plasma of iron-overloaded mice. The most striking increase was observed for MDA in plasma. MDA concentration rose by approximately 6-fold, increasing from 15% of total aldehydes in control animals, to 35% of total aldehydes after 3 weeks of iron treatment. HNE was detected at 20% the concentration of MDA in control plasma, and increased by 2-fold after 3 weeks of iron treatment.

Hepatic Protein Modification and Oxidation During Chronic Iron Administration

The extent of modification of total liver protein by products of lipid peroxidation was used as an indicator of protein damage during chronic iron overload. As shown in Fig 2, there was a gradual accumulation of CML, MDA-Lys, and HNE-Lys in

Table 1. Plasma Aldehyde Profile as a Function of Total Dose of Iron Administered

Aldehydes	Cumulative Dose of Iron Administered (nmol/L)			
	Basal Value	100 mg	200 mg	300 mg
Propanal	75 ± 5†	96 ± 11‡§¶	133 ± 24§	146 ± 17*
Butanal	13 ± 1†	27 ± 12§	37 ± 12	44 ± 9*
Pentanal	34 ± 1¶	41 ± 5§	40 ± 2§	45 ± 6§
Furfural	242 ± 42†‡	364 ± 49†‡§	432 ± 23†	644 ± 40*
Hexanal	27 ± 1†	56 ± 7*†‡	95 ± 11*	118 ± 18*
Trans-2-hexenal	3 ± 1†	6 ± 3‖	10 ± 2*	10 ± 1*
Heptanal	422 ± 49	455 ± 43¶	602 ± 60§	614 ± 42§
Trans-2-heptenal	169 ± 16†	190 ± 39¶‖	242 ± 10*	321 ± 15*‡
Trans, trans-2,4-heptadienal	68 ± 4†	74 ± 8‡¶	131 ± 14*	148 ± 33*
Octanal	356 ± 35	364 ± 46¶	386 ± 34	487 ± 11‖
Trans-2-octenal	99 ± 19¶	138 ± 19‡§¶	185 ± 1*	185 ± 28§
Nonanal	441 ± 73	465 ± 64	516 ± 51	489 ± 49
Trans-2-nonenal	115 ± 25†	224 ± 31†§	238 ± 11*†	367 ± 22*
Trans-2, *cis*-6-nonadienal	52 ± 6†‡	63 ± 12†	71 ± 8	97 ± 9*‡
Trans, trans-2, 4-nonadienal	76 ± 17¶	83 ± 20‡	116 ± 7	113 ± 21§
4-hydroxy-non-2-enal	109 ± 5†‡	147 ± 25§¶	161 ± 24¶	209 ± 10*‖
Decanal	420 ± 51	488 ± 53	494 ± 77	473 ± 61
Trans-4, *cis*-4-decenal	266 ± 27¶	280 ± 34¶	312 ± 28	377 ± 22§
Dodecanal	406 ± 34‡¶	572 ± 19§	540 ± 34	601 ± 74§
Malondialdehyde	568 ± 53†	1,535 ± 279*‡	2,365 ± 220*	3,378 ± 374*‡
Total	3,692 ± 112	5,696 ± 206*	7,070 ± 431*	9,568 ± 1,333*

NOTE. All values are mean ± SD (n = 5).

*$P < .001$ *v* control; †$P < .001$ *v* 300 mg iron; ‡$P < .01$ *v* 200 mg iron; §$P < .05$ *v* control; ¶$P < .05$ *v* 300 mg iron; ‖$P < .05$ *v* 200 mg iron.

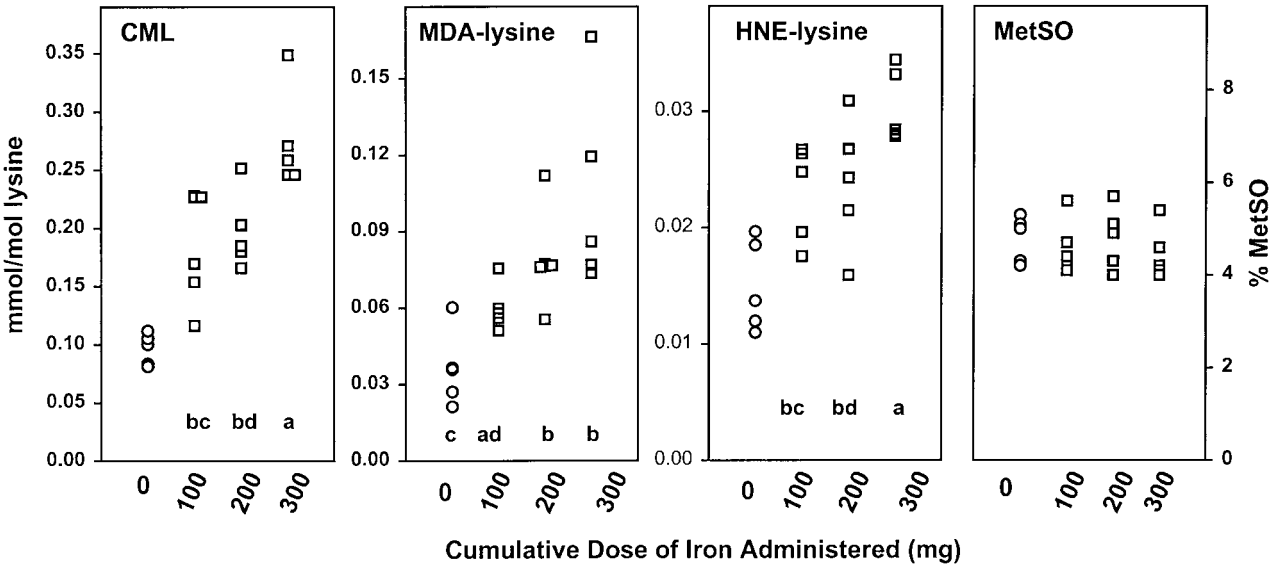

Fig 2. Hepatic concentrations of lipoxidation products, but not MetSO, increase during chronic iron administration to mice. Biomarker analyses were conducted by SIM-GC/MS, as described in the text. Data are values for individual animals (n = 5 per group) at each dose of iron; [a]$P < .001$ compared to control, [b]$P < .01$ compared to control, [c]$P < .01$ compared to 300 mg iron, and [d]$P < .03$ compared to 300 mg iron.

hepatic proteins with increasing iron administration. By the third week of the experiment, the levels of all of the lipoxidation products had increased by approximately 3-fold over baseline values, with strong correlations among these biomarkers: CML versus MDA-Lys or HNE-Lys, $r = 0.87$, and MDA-lysine versus HNE-lysine, $r = 0.78$, all with $P < .0001$. The increases in lipoxidation products were in reasonable agreement with the ratios and fractional increase in concentrations of aldehydes in plasma, ie, the concentration of MDA-Lys was greater than that of HNE-Lys in hepatic proteins, and the relative increase in plasma MDA-Lys was greater than that of HNE-Lys. MetSO is a marker of direct protein oxidation by reactive oxygen species, independent of carbonyl intermediates formed during lipid peroxidation reactions. The basal value for MetSO in liver protein was 4.8 ± 0.5%, expressed as MetSO/(MetSO + Met), and this value remained essentially unchanged throughout the experiment (Fig 2).

Modification and Oxidation of Skin Collagen During Chronic Iron Administration

The accumulation of oxidative damage to long-lived extracellular proteins during iron administration was monitored in insoluble skin collagen. Of the chemical modifications studied in the present experiments, only CML and MetSO have been reported in skin collagen. The data summarized in Fig 3 demonstrate that neither marker was increased substantially until a total of 300 mg of iron had been administered to the animals, but at that point there was a large increase in both markers. There was also a strong correlation between CML and MetSO concentrations in skin collagen ($r = 0.91$, $P < .0001$). These results indicate that, despite the gradual increase in plasma aldehyde content (Table 1), extracellular collagen in skin was relatively resistant to lipoxidative damage, compared to hepatic proteins. The increase in MetSO in collagen is consistent with increased exposure of the protein to oxidative stress during iron overload and the absence of extracellular MetSO reductase activity.

DISCUSSION

Chronic iron overload eventually leads to organ failure, and is found clinically in conditions such as hereditary hemochro-

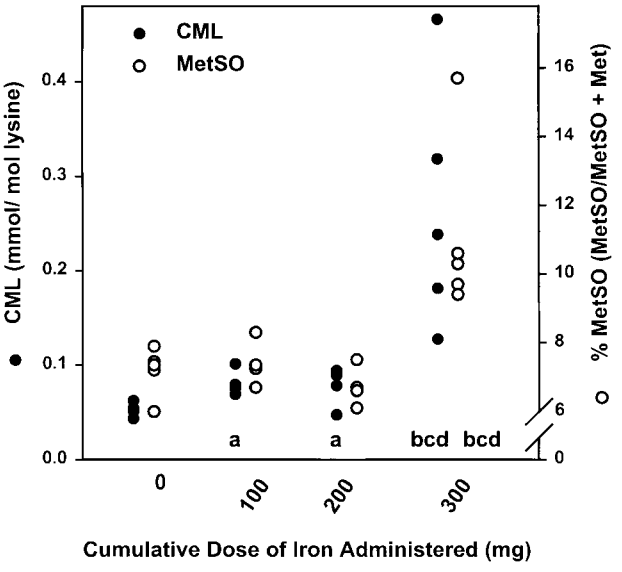

Fig 3. Concentrations of both CML and MetSO are increased in insoluble skin collagen during late stage of chronic iron administration to mice. Data are values for individual animals (n = 5 for all except n = 4 for 100 mg iron) at each dose of iron; [a]$P < .05$ compared to control, [b]$P < .006$ compared to control, [c]$P < .02$ compared to 100 mg iron, and [d]$P < .008$ compared to 200 mg iron.

matosis and sideroblastic anemia, and after prolonged transfusion therapy for the management of β-thalassemia major.[45-47] Free radical damage is hypothesized to be a significant factor in the pathogenesis of iron-overload disease. In support of this hypothesis, our results show that chronic iron-loading leads to significant increases in the plasma concentration of aldehydes derived from lipid peroxidation reactions, and also to an increase in lipoxidation products in hepatic protein. The increases in lipoxidation products were modest in comparison to the increase in total iron stores in liver, indicating the presence of efficient mechanisms for iron sequestration in liver, coupled with potent hepatic antioxidant defenses and dicarbonyl detoxification systems. The simultaneous and comparable increase in lipid-derived aldehydes in plasma and lipoxidation products in liver is consistent with immunohistochemical detection of increased levels of MDA and HNE adducts in liver and plasma proteins of rats maintained on diets containing iron-carbonyl.[14,16,48]

MetSO is recognized as one of the most readily oxidized, surface-exposed amino acids on proteins and is considered to have a defensive role in protection of proteins against oxidative damage.[49] The failure to detect an increase in MetSO in liver proteins is inconsistent with the increase in lipoxidation products, but suggests that *hepatic* antioxidant defenses may not be compromised by iron overload, especially at the extent of iron overload associated with human disease. The lack of an increase in hepatic MetSO content may reflect the activity of hepatic MetSO reductases[50,51]; however, it is also possible that MetSO-containing protein may be more efficiently targeted for degradation. The increase in plasma aldehydes and hepatic lipoxidation products, in the absence of an increase in MetSO, suggests that hepatic carbonyl detoxification systems may be overloaded by the high aldehyde flux. Thus, in contrast to MetSO, aldehydes and lipoxidation products may be more useful as biomarkers of intracellular oxidative stress.[38,39]

In addition to the failure to observe an increase in MetSO in liver, the increase in MetSO (and CML) in skin collagen was significantly delayed. Neither of the biomarkers changed markedly in skin collagen until the end of the study, following administration of large amounts of iron (Fig 3). These observations suggest that skin collagen may not provide good sensitivity for detection of systemic oxidative stress, either because the changes in biomarkers are slow to occur in skin or because they do not occur until a certain threshold of oxidative stress has been reached. The lack of a timely or sensitive response to oxidative stress in skin collagen may affect conclusions regarding the status of oxidative stress in other diseases. For example, the failure to detect significant increases in CML and MetSO in skin collagen of diabetic patients has been interpreted as evidence against a generalized increase in oxidative stress in diabetes.[31,52] The present results suggest that measurement of specific lipoxidation biomarkers (CML, MDA-Lys, HNE-Lys) in intracellular proteins in liver and/or other tissues may provide a more reliable index of the status of oxidative stress in diabetes and the role of oxidative stress in the development of diabetic complications.

During their evolution, mammalian cells have developed antioxidant defenses in an attempt to inhibit free radical-mediated damage. GPx is a major protective selenoenzyme that helps to prevent the generation of free radical species via Fenton-type reactions by scavenging H_2O_2 and other organic peroxides.[53-55] Indeed, it has been demonstrated that the cytotoxicity of H_2O_2 in cultured hepatocytes[56] and cardiac myocytes is iron-dependent.[57,58] Our findings show that chronic iron-loading in a murine model results in dose-dependent decreases in the activity of GPx in liver, perhaps as a consequence of the increase in HNE in liver and plasma, since HNE is known to inhibit GPx activity in vitro.[59] In agreement with the results of these model studies, GPx activity has been shown to decrease following metal toxicosis in other experimental models,[18,37,60,61] and in patients with β-thalassemia and chronic iron overload.[27] Patients with iron-overload disease have also been reported to have increased free radical activity in plasma, with accompanying decreases in antioxidant reserves, including vitamins C and E and erythrocyte GPx activity.[24,27,62] At the same time, the decline in GPx activity in the present experiments was relatively modest compared to the massive accumulation of iron, perhaps because of a compensatory induction of GPx as an endogenous protective response to increased free radical activity in the liver,[18,19,63,64] secondary to chronic iron overload.

In summary, while no single mechanism is likely to account for the complex pathophysiology of chronic iron-induced organ failure, our work demonstrates that iron-loading in a murine model results in dose-dependent increases in lipoxidative damage to proteins in liver and skin. Evidence of oxidative stress, as measured by the concentration of MetSO in proteins, is more readily detected in proteins in the extracellular matrix, or perhaps in plasma proteins, where the damage is irreversible. Further research is necessary to identify reliable intracellular markers of oxidative damage to proteins, but at present the measurement of lipoxidation products appears to provide a useful index of lipid peroxidation in response to iron overload. Application of these assays to analysis to tissues from persons with iron storage diseases should provide quantitative insight into the role of iron and oxidative stress in the pathogenesis of these conditions.

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