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### Research Article

In hepatic iron overload, iron-catalyzed lipid peroxidation has been implicated in the mechanisms of hepatocellular injury. Lipid peroxidation may produce reactive aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), which may form aldehyde-protein adducts. We investigated whether lipid peroxidation occurred in rats fed a diet containing 3% carbonyl iron for 5-13 wk, and if this resulted in the formation of MDA- and 4-HNE- protein adducts. Chronic iron feeding resulted in hepatic iron overload (greater than 10-fold) and concomitantly induced a 2-fold increase in hepatic lipid peroxidation. Using an antiserum specific for MDA-lysine protein adducts, we demonstrated by immunohistochemistry the presence of aldehyde-protein adducts in the cytosol of periportal hepatocytes, which co-localized with iron. In addition, MDA- and 4-HNE-lysine adducts were found in plasma proteins of animals with iron overload. Only MDA adducts were detected in albumin, while other plasma proteins including a approximately 120-kD protein had both MDA and 4-HNE adducts. In this animal model of hepatic iron overload, injury occurs primarily in periportal hepatocytes, where MDA-lysine protein adducts and excess iron co-localized.

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# Malondialdehyde and 4-Hydroxynonenal Protein Adducts in Plasma and Liver of Rats with Iron Overload

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## Abstract

In hepatic iron overload, iron-catalyzed lipid peroxidation has been implicated in the mechanisms of hepatocellular injury. Lipid peroxidation may produce reactive aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), which may form aldehyde-protein adducts. We investigated whether lipid peroxidation occurred in rats fed a diet containing 3% carbonyl iron for 5–13 wk, and if this resulted in the formation of MDA- and 4-HNE- protein adducts. Chronic iron feeding resulted in hepatic iron overload (> 10-fold) and concomitantly induced a 2-fold increase in hepatic lipid peroxidation. Using an antiserum specific for MDA-lysine protein adducts, we demonstrated by immunohistochemistry the presence of aldehyde-protein adducts in the cytosol of periportal hepatocytes, which co-localized with iron. In addition, MDA- and 4-HNE-lysine adducts were found in plasma proteins of animals with iron overload. Only MDA adducts were detected in albumin, while other plasma proteins including a  $\approx$  120-kD protein had both MDA and 4-HNE adducts. In this animal model of hepatic iron overload, injury occurs primarily in periportal hepatocytes, where MDA-lysine protein adducts and excess iron co-localized. (*J. Clin. Invest.* 1990. 86:1991–1998.) Key words: aldehydes • oxidative stress • autofluorescence • lipofuscin • immunochemistry

## Introduction

Hemochromatosis is associated with excess iron deposition in hepatocytes, which results in hepatic injury (1, 2). The mechanisms responsible for the development of hepatocellular injury in patients with hemochromatosis are not known, but a direct correlation between hepatic iron and hepatic fibrosis has been demonstrated (3, 4). Because lipid peroxidation is known to be catalyzed by iron, enhanced lipid peroxidation has been proposed as an initial step by which excess iron causes cellular injury (1, 5). This proposal is supported by results from animal studies and in vitro models (6–12). In rats with chronic iron overload, lipid peroxidation is associated with changes in mitochondrial (7) and microsomal function (11), and with increased lysosomal fragility (12).

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Iron-catalyzed lipid peroxidation of polyunsaturated fatty acids leads to the formation of highly reactive aldehydes, such as malondialdehyde (MDA)<sup>1</sup> and 4-hydroxynonenal (4-HNE), which may then form covalent links to proteins, phospholipids, and DNA (13, 14). In proteins, these links involve the  $\epsilon$ -amino group of lysine residues or sulfhydryl groups (13). Oxidation of LDL has been proposed to play an etiologic role in atherogenesis (15, 16), and MDA- and 4-HNE-modified LDL protein have been found in atheromas of the hyperlipidemic Wantanabe rabbit (17–19), and in man (18). Analogously, acetaldehyde, the first metabolite of ethanol, has been shown to produce adducts with proteins in vivo, judging by the presence of antibodies against the acetaldehyde-lysine epitope in the sera of alcoholic patients (20) and the identification of a protein-acetaldehyde adduct in liver homogenates of ethanol-fed rats (21).

In this study, we fed rats excess iron (22) and demonstrated the presence of MDA-lysine adducts in the cytosol of periportal hepatocytes, co-localized with iron. In addition we demonstrated the presence of MDA- and 4-HNE-lysine adducts in several plasma proteins.

## Methods

**Materials.** Nitrocellulose BA85, 0.45  $\mu$ m was purchased from Schleicher & Schuell (Keene, NH). Sources of other chemicals were: 3,3'-diaminobenzidine tetrahydrochloride dihydrate (DAB), from Aldrich Chemical Co. (Milwaukee, WI); gelatin Type III, carbonyl iron (99% elemental iron in particles ranging from 4.5–5.2  $\mu$ m), and horse spleen ferritin from Sigma Chemical Co. (St. Louis, MO); immunoelectrophoresis tricine buffer IV, pH 8.6 from Bio-Rad Laboratories (Richmond, CA); EM-bed 812 from Electron Microscopy Sciences (Fort Washington, PA); biotinylated antibodies, Vectastain kits and reagents from Vector Laboratories, Inc. (Burlingame, CA); rabbit anti-rat albumin, IgG fraction, rat albumin, and rabbit anti-transferrin from Cappel Laboratories (Malvern, PA); Histo-Clear from National Diagnostics, Inc. (Manville, NJ); rabbit anti-human ferritin from Dako Corp. (Carpinteria, CA); and <sup>125</sup>I-protein A from Amersham Corp. (Arlington Heights, IL).

**Animal procedures.** Sprague-Dawley male rats (100–125 g) (Charles River Breeding Laboratories, Inc., Wilmington, MA) were pair fed a 3% (wt/wt) carbonyl iron diet (6) or control, 5001 rat chow diet (Ralston-Purina Co., Richmond, IN). Animals were weighed weekly and the diet of the control animals adjusted to maintain similar growth rates to the carbonyl iron-fed animals. At 5 wk, animals were anesthetized by an intramuscular injection of ketamine (100 mg/kg), zylazine (2 mg/kg), and acepromazine (2.5 mg/kg) and then killed by cardiac puncture and their livers promptly removed. A portion was rinsed in ice-cold PBS and immediately frozen in liquid nitrogen; the remainder was fixed in 10% formalin for Perls' prussian blue staining.

1. **Abbreviations used in this paper:** BHT, butylated hydroxytoluene; DAB, 3,3' diaminobenzidine tetra hydrochloride dihydrate; EITB, electroimmunoblot; 4-HNE, 4-hydroxynonenal; MDA, malondialdehyde; TBARS, thiobarbituric reactive substances.

A separate group of animals was maintained on either a 3% carbonyl iron diet or control diet for 13 wk. The livers were biopsied under anesthesia (as described above) while still perfused, and immediately fixed as described below for immunohistochemistry; the remainder of the liver was removed and processed as described above. In some animals, the liver was fixed in Karnovsky's fixative overnight at 0–4°C. The tissue was postfixed in 2% osmium tetroxide for 1 h at 20°C and then dehydrated through a graded series of ethanol and propylene oxide and embedded in EM-bed 812. Thin sections (60 nm) were cut and stained with uranyl acetate and bismuth subnitrate.

**Iron determination.** Non-heme iron content was measured using the method of Horak et al. (23) except that liver was used in place of plasma as described by Torrance and Bothwell (24). Values are expressed as micrograms of iron per gram liver (wet weight).

**Thiobarbituric acid reactivity.** Thiobarbituric acid reactive substances (TBARS) were determined from liver specimens stored in liquid nitrogen as described by Ohkawa and co-workers (25) except that SDS was omitted and EDTA (3 mM) was added to the tissue homogenization solution. The fluorescence of the color complex formed was measured (excitation 515 nm and emission 553 nm) and compared to standards prepared from tetramethoxypropane. To exclude the possibility that spurious TBARS were formed in the assay conditions due to excess iron (26), iron in the form of ferritin was added to samples of normal liver, before homogenization, in an amount equal to that found in iron-overloaded rats.

**Antisera.** Monospecific antisera to MDA- and 4-HNE-lysine adducts were generated using techniques previously described (17, 18, 27, 28). In brief, guinea pig LDL was isolated and modified with MDA or 4-HNE, and the homologous modified LDL were used to immunize guinea pigs (17, 18, 28). The resultant antisera were specific for the adducts to LDL and did not react with native LDL. Each antiserum is epitope specific and reacts with this adduct on a variety of different proteins. Thus "MAL-2" is specific for MDA-lysine adducts (17, 28) and antiserum "4-HNE" is specific for the 4-HNE-lysine adduct (28). Previous reports using these antisera have been published (17, 18, 28).

**Immunohistochemistry of hepatic tissue.** Portions of the liver from pair-fed animals (control,  $n = 3$ ; and carbonyl iron fed,  $n = 3$ ) were cut into  $2 \times 4$  mm sections and prepared as described by Palinski and colleagues (17). EDTA and butylated hydroxytoluene (BHT) were added to all solutions to prevent spurious lipid peroxidation during sample preparation (26). A minimum of three 5- $\mu$ m sections of at least two specimens per liver were placed on gelatin-coated slides and deparaffinized using Histo-clear, passed through graded series of alcohol, and subsequently rehydrated in 0.15 M NaCl, 50 mM sodium phosphate, pH 7.5 (PBS). Immunohistochemistry was performed using an avidin-biotin-alkaline phosphatase system (Vector Laboratories, Inc.) as described by the manufacturer. Sections were immunostained with antiserum MAL-2 (1:500), 4-HNE (1:1,000), preimmune serum (1:500), or PBS. The brown-black color produced was used to assess the presence of MDA-lysine and 4-HNE-lysine protein adducts.

Adjacent liver sections were deparaffinized and rehydrated as described above and then mounted using glycerol-PBS (9:1), pH 8.2. Autofluorescence was detected using either an ultraviolet, fluorescein, or rhodamine excitation filter. Subsequently, after photographing the autofluorescence, the cover slips from the same slides were removed and the sections rinsed in PBS (pH 7.4) and stained for iron as described above.

**Electroimmunoblotting.** Plasma from rats fed control and iron diets for 5 wk was obtained by cardiac puncture using an EDTA-coated syringe. Samples were immediately chilled to 0°C and additional EDTA was added to make the final concentration 3 mM. Plasma was separated by centrifugation at 0°C and stored in liquid nitrogen until used for electroimmunoblotting (EITB). To determine whether nontransferrin-bound iron was important in the formation of circulating aldehyde-protein adducts and not excessive storage iron, the following experiments were performed. In pair-fed animals the carbonyl iron diet was replaced with control diet for 5 d. Plasma was obtained, as described above, before and after stopping the carbonyl iron diet and

used for EITB. Additionally, plasma from rats fed the control diet was obtained using heparin, in place of EDTA, to coat the syringe. Ferric chloride (in 50 mM citrate buffer, pH 7.4) was added to aliquots of plasma to increase the concentration of iron by 2 or 6  $\mu$ g/ml and incubated at 25 or 37°C for 1 h. EDTA (10 mM final) was added to all plasma samples to stop the reaction, and the treated plasma was used for EITB immediately.

Plasma proteins were separated by SDS-PAGE (29), using a 3% stacking and 7.5% resolving gel. Samples were not reduced before separation, since this was found to decrease antigen detection by Western blot (Houglum, K., and M. Chojkier, unpublished observations) (17, 18). Some gels were stained with Coomassie blue before or after transblotting to nitrocellulose as described by Towbin (30). Primary antibodies were applied in the following dilutions in 10 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween 20: MAL-2, 1:400; 4-HNE, 1:200; rabbit anti-rat albumin, IgG fraction, 1:400; rabbit anti-rat transferrin, 1:200. Biotinylated secondary antibody, avidin-biotin-peroxidase system, and DAB reactions were performed as described by the manufacturer. In some experiments  $^{125}$ I-labeled protein A (10  $\mu$ Ci) was used instead of secondary antibody.

The presence of albumin dimers and trimers in the plasmas of control and iron-fed animals was verified by running the plasma proteins on a SDS-PAGE gel as described above. The gel was divided into two halves that contained identical sample lanes. One-half was processed as described above and stained for albumin. The regions in the untreated half, which corresponded to those areas that stained for albumin, were excised and run on a 1% agarose gel, against an albumin standard, in 25 mM tricine-Tris, pH 8.6.

**Two-dimensional SDS-PAGE.** Isoelectric focusing of the plasma proteins was described by O'Farrell (31). For the second dimension the gels were then run into a 7.5% SDS-PAGE gel and immunostained as described under EITB.

**Ferritin and hemosiderin isolation.** Ferritin and hemosiderin in livers from control ( $n = 3$ ) and carbonyl iron-fed rats ( $n = 3$ ) were isolated as described by Weir (32). Purity of the sample was ascertained on 12.5% SDS-PAGE after reduction. In the iron-fed rats, most of the purified proteins were ferritin (determined from the 20-kD band) and the remainder, hemosiderin (determined from the 14.5-kD band), with ferritin and hemosiderin accounting for more than 90% of the proteins. This purified ferritin and hemosiderin, horse spleen ferritin, MDA-LDL were subjected to dot blot analysis. MDA-LDL was prepared as described before (17, 28). Duplicate blots were stained with MAL-2 (1:400) or rabbit anti-human ferritin (1:400) as described in EITB.

**Statistical methods.** All the results are expressed as mean  $\pm$  SEM. The Student's  $t$  test was used to evaluate the difference of the means between groups, accepting  $P < 0.05$  as significant (33).

## Results

**Hepatic iron concentration.** Rats fed a carbonyl iron diet for 5 wk attained a moderate degree of iron overload that was 13-fold greater than diet-restricted controls (Table I). The body weight was similar in the two groups. The iron concentration in the iron-fed rats was lower than that reported by Park et al. (22), but this may be due to our use of older animals (100–125 g). Fig. 1 shows stainable iron in the liver of a control rat and a rat fed the carbonyl iron diet for 13 wk (3,900  $\mu$ g iron/g liver). The iron was present in all periportal areas (zone 1), and virtually absent around the terminal hepatic venules (zone 3). Iron was found in hepatocytes and distributed in a pericanalicular pattern.

**Thiobarbituric acid reactivity.** (TBARS), a measure of lipid peroxidation (25, 26), was significantly increased in liver homogenates of animals fed the 3% carbonyl iron diet (Table I). The addition of ferritin (final concentration 1,300  $\mu$ g iron/g liver) during sample preparation did not increase the produc-

Table 1. Hepatic Iron Concentration, TBARS, and Body Weight in Iron-Overloaded Rats

Experimental group*	Iron $\mu\text{g/g wet wt}$	TBARS $\text{nmol/g}$	Body weight $\text{g}$
Control ( $n = 5$ )	104 $\pm$ 6	35 $\pm$ 2	263 $\pm$ 9
Carbonyl iron ( $n = 5$ )	1350 $\pm$ 130 <sup>†</sup>	74 $\pm$ 3 <sup>†</sup>	272 $\pm$ 14

\* Animals (100–125 g) were fed either a Purina chow (Ralston-Purina Co.) (control) or a 3% carbonyl iron diet for 5 wk. Hepatic iron and TBARS were measured as described in Methods; all values are mean $\pm$ SEM. <sup>†</sup> Carbonyl iron fed versus control;  $P < 0.05$ .

tion of TBARS by normal liver (data not shown), suggesting that the increased concentration of TBARS in livers of iron-fed rats reflects in vivo formation. Similarly, Bacon et al. (6) have reported increased conjugated dienes in the mitochondria of animals with comparable iron overload to ours.

**Immunohistochemistry of hepatic tissue.** We have previously generated an antiserum, MAL-2, that specifically recognizes MDA-lysine residues present on a variety of different

proteins (17, 18, 28). A fluorescent probe was chosen to enhance the detection of specific MAL-2 staining in hepatocytes and siderotic nodules that contained a significant amount of dense hemosiderin pigment, which we thought might mask the staining by enzymatic methods. However, what was found was an intense yellow-orange autofluorescence in untreated sections (Fig. 2, B and C). The distribution of this autofluorescence was predominately in periportal hepatocytes (zone 1) and co-localized to the same hepatocytes with stainable iron in the identical tissue sections (Fig. 2, B and C and Fig. 1 B). Autofluorescence was absent around the terminal hepatic venule (zone 3) in iron-overloaded livers (Fig. 2, B and C) and also in liver sections obtained from control animals (Fig. 2 A). To our knowledge, autofluorescence in experimental iron overload has not previously been described, however Lillie and Fullmer (34) suggested that a non-hemosiderin yellow pigment, that has been described in patients with hemochromatosis, is lipofuscin. Lipofuscin contains fluorophores, which are products of lipid peroxidation (35). Indeed, lipofuscin as detected by its characteristic electron microscopic pattern (36) was abundant in the periportal hepatocytes of iron-overloaded animals (Fig. 2 D) but rare in controls (data not shown). Periportal (zone 1) hepatocytes showed a diffuse as well as vesicular pattern of MAL-2 reactivity that diminished in zone 2 and was absent around the terminal hepatic venules (zone 3) (Fig. 3 B). The vesicular pattern was most prominent in the cytoplasm of hepatocytes and no nuclear staining was identified (Fig. 3 B). Staining for the MDA-lysine epitope (Fig. 3 B) and iron (Fig. 1 B) co-localized to zone 1 hepatocytes, but neither was present in zone 3 hepatocytes. Hemosiderin, a dense yellow pigment in immunostained sections, can be seen in Fig. 3 B. The sections shown in Fig. 3 B were obtained from an animal with a hepatic iron load of 3,900  $\mu\text{g/g}$  of liver and are representative sections. Adjacent sections incubated with preimmune serum or PBS showed no specific staining, however sinusoidal staining was still apparent and not diminished by the alkaline phosphatase inhibitor levamisole. Some liver sections obtained from control animals showed a rare hepatocyte with staining, among multiple portal triads viewed. This may represent either nonspecific staining or specific staining in degenerating hepatocytes. The same pattern of nonspecific sinusoidal staining, as seen in section of iron-loaded liver, was also demonstrated in control liver sections (Fig. 3 A). No difference was seen between control and iron-fed animals in sections stained with 4-HNE (data not shown). The reason for the difference between 4-HNE and MAL-2 staining is not clear. In other experiments using  $\text{CCl}_4$ -intoxicated animals, both MDA- and 4-HNE-protein adducts in the liver are seen in the same zonal distribution (37), however 4-HNE consistently stained less intense (Houglum, K. and M. Chojkier; unpublished observations). This may reflect a difference in the sensitivity of the antisera or a less abundant antigen.

Ferritin and hemosiderin isolated from iron-overloaded animals had no detectable MDA-protein adducts (data not shown). Therefore, it seems unlikely that the MDA-protein adducts seen in Fig. 3 B represent either ferritin or hemosiderin.

**Plasma adducts.** Because reactive aldehydes formed by lipid peroxidation in iron-overloaded hepatocytes were shown to modify hepatic proteins, we also investigated whether MDA- or 4-HNE-lysine adducts were present in the plasma of iron-overloaded rats. Plasma was collected in the presence of

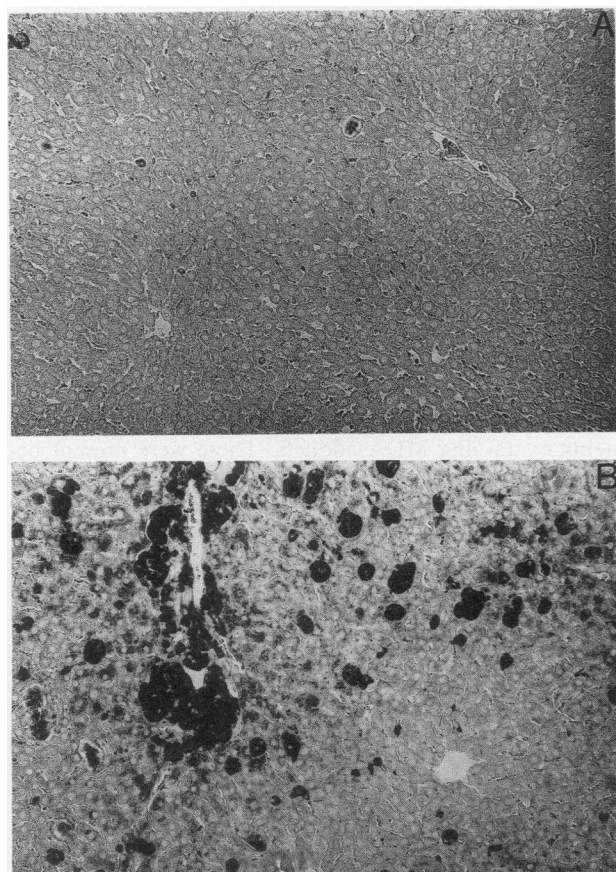
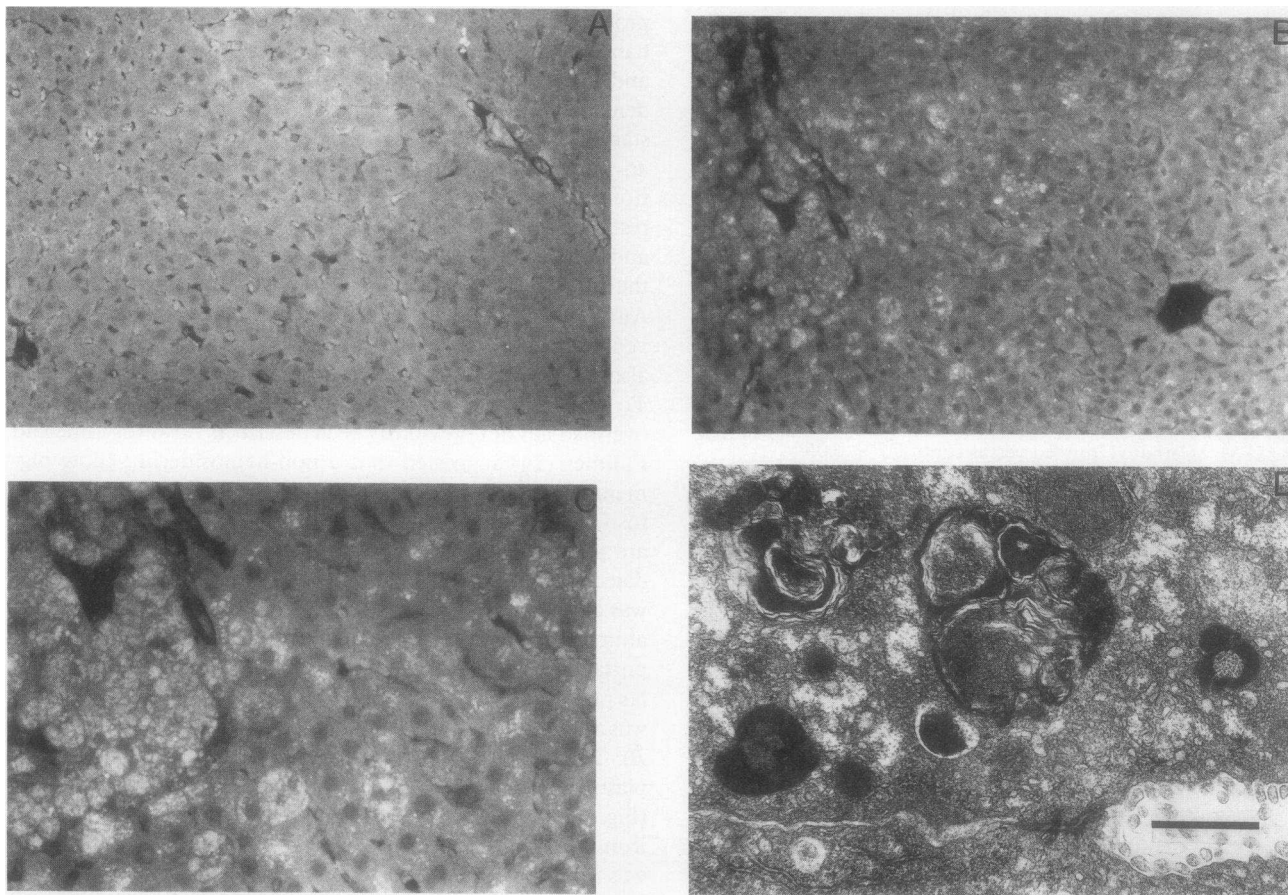


Figure 1. Perls' Prussian blue staining of liver. Paraffin sections of liver from a control (A) and an iron-overloaded rat (3,900  $\mu\text{g/g}$  wt) (B) were treated with Perls' Prussian blue, which stains for iron (80 $\times$ ).

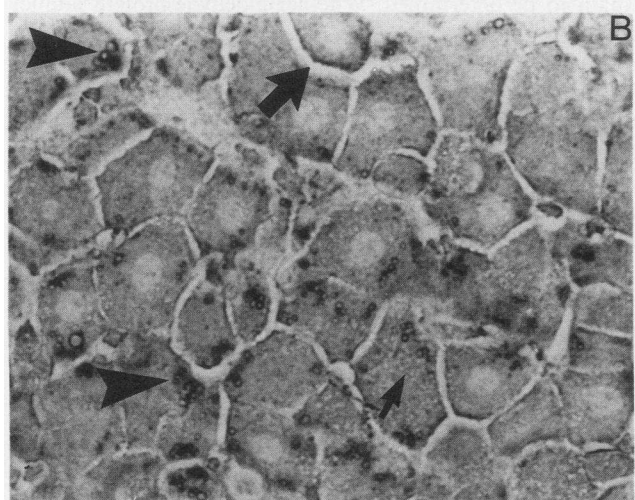
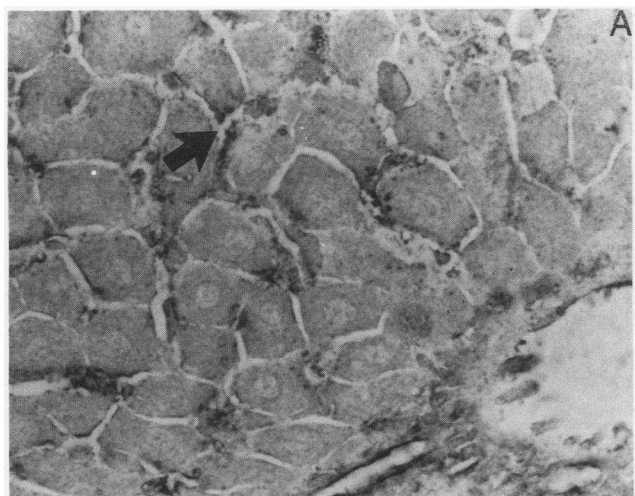


**Figure 2.** Co-localization of autofluorescence and iron. Liver sections (used in Fig. 1) from a control (*A*) and iron-overloaded rat (3,900  $\mu\text{g/g}$  wet wt) (*B* and *C*) were prepared as described in Methods. Autofluorescence was detected using a rhodamine excitation/emission cube. A higher magnification of *B* is shown in *C* (*A* and *B*, 120 $\times$ ; *C*, 240 $\times$ ). The electron micrograph (*D*) demonstrates typical lipofuscin particles which were abundant in the periportal hepatocytes of iron overloaded rats (bar is 1  $\mu\text{m}$ ).

excess EDTA in order to prevent lipid peroxidation and the formation of aldehyde adducts during the processing of samples (26, 28). Plasma containing an equal amount of protein from both control and iron-fed rats was subjected to SDS-PAGE and then used for Western blotting with antiserum MAL-2. Western blots of plasma proteins from the control rats were negative with MAL-2 when stained with DAB-peroxidase. In contrast, MAL-2 consistently reacted with several different protein bands prepared from the iron-overloaded rats (Fig. 4). In subsequent experiments, even using the more sensitive  $^{125}\text{I}$ -labeled protein A method, the 120-kD band was minimally detected in control animals (data not shown). The lowest molecular weight protein detected had a  $\approx$  66 kD mol wt, similar to albumin. Fig. 5 shows the results of a Western blot of plasma from an iron-overloaded rat in which the nitrocellulose was divided into two parts and half probed with the MAL-2 antiserum and the other half exposed to antiserum specific for rat albumin. Several bands were positive for albumin, at 66 kD and between 92 and 200 kD, suggesting that albumin monomers, dimers, and higher molecular weight aggregates were present. These bands also reacted with MAL-2. However, there were additional proteins that had the MDA-lysine epitope, between 66 and 92 kD. The existence of albumin dimers and trimers was confirmed first by isolating the putative polymers of albumin by SDS-PAGE gel electrophore-

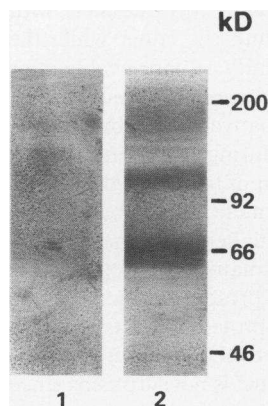
sis and then by running them on a 1% agarose gel. Identical mobility, compared with authentic rat albumin, indicated that some of the high molecular weight proteins were indeed albumin (data not shown). Two-dimensional gel electrophoresis confirmed that albumin contained MDA-lysine adducts (see arrow, Fig. 6), but also identified the presence of several other proteins containing MDA adducts. These were present between 66 and 200 kD and had different isoelectric points than that of native albumin. Possibly, some of the higher molecular weight proteins were albumin aggregates whose isoelectric point was altered by the MDA modification.

Western blots of plasma proteins were also done with the antiserum specific for 4-HNE. Proteins from normal rats showed minimal reactivity at  $\approx$  120 kD, compared with several different bands that were identified in the proteins of iron-overloaded rats (Fig. 7). The presence of 4-HNE adducts like MDA adducts in normal plasma was not unexpected, and most likely represents the presence of 4-HNE-lysine adducts under normal conditions (17). The marked increase in 4-HNE-lysine adducts present in iron-loaded animals probably represents the enhanced formation of reactive 4-HNE under experimental conditions. A direct comparison of the protein adducts recognized by antisera MAL-2 and 4-HNE is shown in Fig. 8. Note that antiserum 4-HNE recognizes some of the same modified proteins as MAL-2 but does not recog-

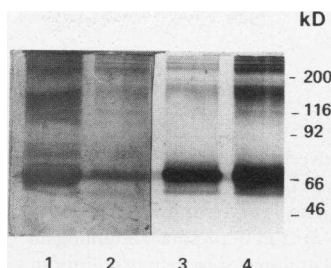


**Figure 3.** Co-localization of MDA-protein adducts and iron. Paraffin embedded hepatic tissue was immunostained with MAL-2, as described in Methods. Control (A) and iron overloaded rats (3,900  $\mu\text{g}$  iron/g wet wt) (B) are shown (A and B, 480 $\times$ ). The arrowheads in B demonstrate the vesicular staining pattern, and the arrows in A and B, the nonspecific sinusoidal staining. Hemosiderin is shown by the small arrow in B.

nize albumin. The amount of MDA- and 4-HNE protein adducts present in the plasma of animals taken off the carbonyl-iron diet for 5 d was unchanged (data not shown). This sup-

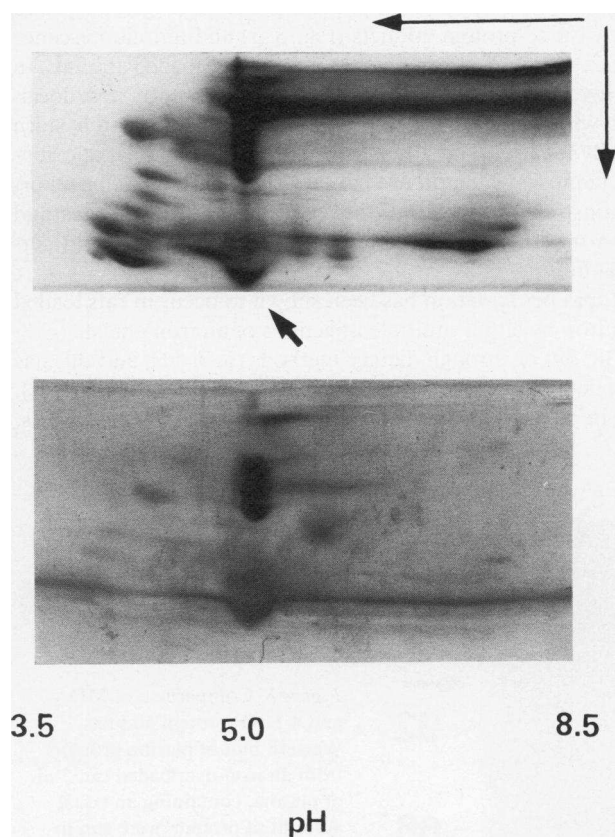


**Figure 4.** MDA-lysine adducts in an iron-overloaded rat. Western blot analysis of plasma proteins from control (lane 1) and iron overloaded rats (lane 2). 2  $\mu\text{l}$  of plasma, containing an equal amount of protein, were run in each lane on a 7.5% SDS-PAGE gel, transferred to nitrocellulose, and immunoblotted with antiserum MAL-2, as described in Methods. Molecular weight standards are shown.

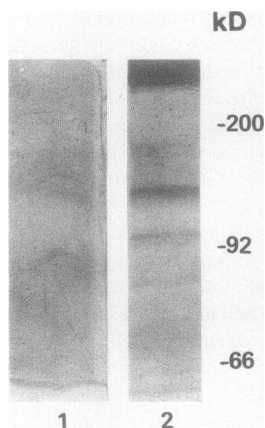


**Figure 5.** Comparison of MDA-lysine adducts to albumin. Western blot of plasma proteins from an iron-overloaded rat. Different dilutions of plasma were run on a 7.5% SDS-PAGE gel; undiluted (lane 1), 1:20 (lane 2), 1:50 (lane 3), and 1:20 (lane 4). The gel was transferred to nitrocellulose and immunoblotted with antiserum MAL-2 (lanes 1 and 2) and antiserum specific for rat albumin (lanes 3 and 4), as described in Methods. Molecular weight standards are shown.

ports the hypothesis that increased storage iron and not high ambient non-transferrin or transferrin-bound iron in the plasma contribute to the formation of aldehyde adducts in vivo. In addition, no increase in MDA- or 4-HNE-protein adducts were found by the in vitro addition of excess iron in the absence of EDTA, suggesting the enhanced formation of these adducts seen in the plasma of iron-overloaded rats occurs in vivo (data not shown). In particular, one protein band with a  $\approx 120$  kD mol wt was consistently identified in the plasma of rats with iron overload by both antisera MAL-2 and 4-HNE



**Figure 6.** Two-dimensional map of MDA-protein adducts. Two-dimensional gel electrophoresis of plasma proteins from an iron-overloaded rat. Isoelectric focusing was performed in the first dimension from pH 3.5–8.5. The second dimension was run on a 7.5% SDS-PAGE gel, transferred to nitrocellulose and immunoblotted with antiserum MAL-2 (bottom), and stained with Coomassie blue (top), as described in Methods. The arrow of the top indicates the position of native albumin, as compared with a purified albumin standard.



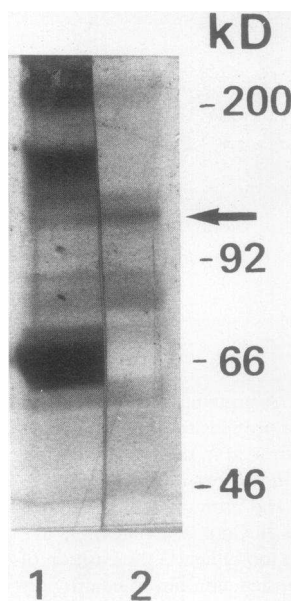
**Figure 7.** 4-HNE-lysine adducts in an iron overloaded rat. Western blot analysis of plasma proteins from control (lane 1) and iron-overloaded rats (lane 2). 2  $\mu$ l of plasma containing an equal amount of protein, were run in each lane on a 7.5% SDS-PAGE gel, transferred to nitrocellulose, and immunoblotted with antiserum 4-HNE (lanes 1 and 2), as described in Methods. Molecular weight standards are shown.

(Fig. 8). Transferrin, which has a 70 kD mol wt, does not correspond with any of the plasma proteins stained with MAL-2 or 4-HNE and furthermore, high molecular weight dimers or trimers of transferrin were not identified in the plasma of iron-overloaded rats (data not shown).

## Discussion

In this study, we demonstrated the presence of MDA- and 4-HNE-lysine adducts in plasma proteins (Figs. 4–8), and MDA-lysine protein adducts (Fig. 3 B) and autofluorescence (Fig. 2, B and C) in hepatocytes of iron-overloaded animals. In the liver, aldehyde adducts and autofluorescence were localized within periportal (zone 1) hepatocytes, which stain heavily for iron, and absent around the terminal hepatic venules (zone 3) which do not contain stainable iron. This report demonstrates co-localization of iron-loaded hepatocytes and MDA-modified protein adducts that resulted from lipid peroxidation.

Lipid peroxidation has been shown to occur in rats loaded with iron by either multiple injections of an iron-chelate (6, 8, 10, 38, 39) or through dietary methods (6, 7, 11), and this has been demonstrated in whole animals (10, 39), in the liver (38), and in subcellular organelles (6, 7, 8, 11). In these reports,



**Figure 8.** Comparison of MDA- and 4-HNE-protein adducts. Western blot of plasma proteins from an iron-overloaded rat. 2  $\mu$ l of plasma, containing an equal amount of protein, were run in each lane on a 7.5% SDS-PAGE gel and transferred to nitrocellulose. The nitrocellulose was divided in half and immunostained with antiserum MAL-2 (lane 1) and 4-HNE (lane 2), as described in Methods. The arrow points to the  $\approx$  120-kD band. Molecular weight standards are shown.

several methods of detecting lipid peroxidation were used, including the measurement of ethane and pentane (10, 39), conjugated dienes (6, 7, 11), and TBARS (8, 38). In cell-free systems, highly reactive aldehydes, formed as a result of lipid peroxidation, can bind to proteins and inhibit their function (40). In addition, because such aldehydes can also bind to DNA (14), they presumably can disrupt nuclear events as well. In acute liver injury induced by carbon tetrachloride in rats, another model in which enhanced lipid peroxidation occurs, an increased number of carbonyl functions were found in phospholipids and proteins (41, 42). In this study, the association between excess iron, TBARS in whole liver homogenates and the co-localization of MDA-lysine adducts, and excess iron in selected hepatocytes strongly suggest that intracellular lipid peroxidation is enhanced by the excess iron, and that reactive aldehydes are formed. The subcellular localization of these adducts and the identification of the affected protein(s) remain to be determined. Whether aldehyde-protein adduct formation leads to cellular injury is not known.

Similarly, modification of arterial wall proteins and LDL by lipid peroxidation has been implicated in the pathogenesis of atherosclerosis (15–18). MDA and 4-HNE adducts have been found in LDL isolated from the atheromas of the Watanabe rabbit and in man (17, 18). These adducts were found in the arterial wall, both extracellularly and within macrophages (17) as well as in LDL (17, 18), but have not been detected to date in circulating LDL (17, 18, 19). In addition, antibodies to these adducts have been found in serum of rabbits and man (17). In a similar manner, formation of acetaldehyde-protein adducts, which occur as a result of acetaldehyde generation during ethanol metabolism, has been proposed as one of the mechanisms by which alcohol injures the liver (43). A 37-kD acetaldehyde-protein adduct has been identified in livers of rats fed a diet containing alcohol (21) but has not yet been characterized. Antibodies to the acetaldehyde-protein epitope have been found in sera from patients with alcoholic hepatitis (20), and in animals fed a diet containing alcohol (44), as well as in normal individuals (20). Acetaldehyde-protein adducts and MDA- and 4-HNE-lysine adducts are likely to be only examples of many different aldehyde-protein adducts found as a result of lipid peroxidation, and it is probable that autoantibodies to many of these neoantigens may occur (27). The relevance of such autoantibodies to the hepatic injury that occurs with iron overload or other situations where lipid peroxidation is so enhanced, remains to be determined.

We also demonstrated the presence of MDA and 4-HNE adducts with plasma proteins in iron-overloaded rats (Figs. 4–8). Most likely, these plasma proteins form adducts with aldehydes at the time of hepatic synthesis, either while the protein is being processed intracellularly or conceivably after secretion into the extracellular compartment (within the hepatic or systemic circulation). Alternatively, they may represent modified proteins that leak out during cell injury. Present data would not support the formation of the aldehyde-protein adducts in the extracellular compartment, since the addition of iron in a form found in patients with hemochromatosis (45) did not enhance *in vitro* adduct formation. In support of a hepatocellular origin for aldehyde-protein adducts, is the stable presence of 4-HNE and MDA-protein adducts found in the circulation of animals despite removal of the iron diet. The presence of an aldehyde-albumin adduct is not surprising since albumin is the predominant plasma protein, and it is synthe-

sized exclusively by the hepatocyte. It is of interest that only MDA-lysine and not the 4-HNE-lysine epitope was identified with albumin. This difference could be methodological and due to an enhanced ability to detect MDA-lysine because of a more sensitive antiserum. Alternatively, the difference could reflect the high reactivity of 4-HNE (13), that might react with proteins within a cellular compartment near to its site of formation, which does not contain albumin. MDA, however, is freely diffusible (46), and may be more widely distributed in both the intra- and extracellular compartments. With the exception of albumin, the pattern of proteins containing MDA and 4-HNE adducts is similar (Fig. 8). Of particular interest is a prominent  $\approx$  120-kD protein. Further work will be required to assess the biological and clinical relevance of the  $\approx$  120-kD protein as well as the other plasma protein adducts. The detection of such adducts might be useful as a noninvasive marker of the disease process and allow one to judge the adequacy of therapy in patients with iron overload.

The demonstration in this study that MDA-lysine adducts in the liver co-localize with iron provides strong evidence for the occurrence of iron-catalyzed lipid peroxidation in vivo. Characterization of the plasma and hepatocyte proteins that are modified may provide clues to clarify the mechanism by which iron overload leads to cellular injury. Our laboratory has previously shown that the highly reactive aldehydes that form as products of lipid peroxidation as well as acetaldehyde from the oxidation of ethanol, stimulate collagen gene expression in cultured fibroblasts (47, 48). We have proposed that lipid peroxidation could be a link between tissue injury and tissue fibrogenesis (48). Furthermore, aldehyde adducts with DNA occur in vitro (14), and they have been implicated in the pathogenesis of some cancers (49). Whether the basis of carcinogenesis in hemochromatosis is linked to this intriguing process remains to be elucidated. Lipid peroxidation and the resultant formation of aldehyde adducts, as those identified in this study, may have a role in these processes which ultimately leads to hepatic injury, fibrosis, and hepatocellular carcinoma.

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