Iron Mediates Production of a Neutrophil Chemoattractant by Rat Hepatocytes Metabolizing Ethanol

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Abstract

Ethanol metabolism in hepatocytes is accompanied by release of a potent lipid chemoattractant for neutrophils. Production of the factor may initiate the inflammation associated with alcoholic hepatitis. In previous studies with a cytosol system from liver, production was blocked by iron chelators as well as by catalase and superoxide dismutase, suggesting the involvement of oxyradicals in formation of the chemoattractant. These studies have examined the role of iron in intact hepatocytes using cells from rats fed an iron-deficient diet, a control diet or a diet containing 3% carbonyl iron. The iron content averaged 1.4 nmol/mg protein in iron-deficient cells, 6.3 in controls and 135.3 in iron-loaded cells. Hepatocytes from all groups were established in primary culture and incubated with ethanol (10 mM); the medium was assayed for chemoattractant activity for human neutrophils. Cultures from chow-fed or iron-loaded animals produced chemoattractant as previously reported. By contrast, chemoattractant production was undetectable in the iron-deficient cultures. Addition of ferric citrate (10 μM) restored chemoattractant production while increasing cellular iron in the deficient cells less than 50% (to 2.3 nmol/mg protein). Addition of desferrioxamine mesylate to cultures of iron-loaded cells ablated chemoattractant production. The data provide evidence for the importance of hepatocellular iron in production of this alcohol-related lipid chemoattractant and suggest that a small intracellular pool of "free" iron plays a critical role. (*J. Clin. Invest.* 1991. 87:45–49.) Key words: desferrioxamine • lipid peroxidation • liver disease

Introduction

Chemotaxis (that is, directional movement of cells in response to a soluble stimulus) appears to be intrinsic to inflammation, accounting for the migration of leukocytes to specific sites. A number of chemoattractants have been identified including leukotrienes (1), extracellular matrix proteins (2), and complement-derived peptides (3). Some not only elicit migration of leukocytes but also stimulate the release of cytotoxic granule contents (4). Inflammation is prominent in alcohol-induced liver injury, characteristically involves neutrophils, and denotes clinically severe disease. We reported previously that hepatocytes produce a lipid chemoattractant as a byproduct of ethanol metabolism and postulated that the chemoattractant is responsible for migration of leukocytes into the liver during alcohol consumption (5). Human, as well as rat, hepatocytes produce the factor in response to ethanol (6), and the chemoattractant is measurable in the blood of rats consuming an ethanol-containing diet (Roll, F. J., unpublished data). With respect to the lack of hepatic inflammation in rats consuming ethanol, we have shown that rat leukocytes, unlike human cells, do not respond chemotactically to the factor (6).

Production of the factor depends on ethanol metabolism: acetaldehyde appears to be a key intermediate, possibly as a generator of oxyradicals within the cell (7, 8). In a cell-free ethanol-metabolizing system, production of the factor was blocked not only by interrupting ethanol metabolism (with 4-methylpyrazole) but also by scavengers of oxygen radicals and iron chelators. The latter data suggest that the chemoattractant formed in vitro arises via a lipid peroxide intermediate (9).

To validate the in vitro findings in an intact system and to explore more generally the reported involvement of excess hepatocellular iron in alcohol-mediated liver disease (10), we undertook studies at the cellular level, using hepatocytes from iron-deficient, control, or iron-loaded rats. The effects of acute iron repletion or depletion in culture also were studied. The results indicate that, in intact hepatocytes, the level of iron is an important determinant of chemoattractant production.

Methods

Animal models. All animals used were weanling male Sprague-Dawley rats. The iron-deficient diet contained < 4 ppm iron (Teklad Test Diets, Madison, WI) and was given with double-distilled water ad lib; animals were housed in mesh bottom cages (11). Control animals received the same diet with added iron (48 ppm) or were fed chow (Ralston-Purina Co., St. Louis, MO; 198 ppm iron). For iron loading, Purina chow was prepared with 3 g/100 g added carbonyl iron. Animals were maintained under 12-h light-dark cycles and were weighed weekly. Iron-loaded animals were fed the carbonyl-iron containing diet for 6–8 wk before being killed.

Iron deficiency was assessed by hemoglobin assay using the cyanomethemoglobin method (Sigma Chemical Co., St. Louis, MO). Animals developed iron deficiency within 3 wk exhibiting an average hemoglobin of 6 g %. Liver ferritin at this time is ~ 10% of the level in controls (12). At the time of death, iron-deficient animals weighed 110–130 g, or 20–30% less than iron-fed controls. Iron-loaded animals weighed 20–30% less than chow-fed controls in line with previous reports of this model (13).

Hepatocyte culture. The liver was dispersed with collagenase, and hepatocytes were purified by centrifugal elutriation, as described previously (14). The initial isolate was > 98% hepatocytes with 90–95% viability as assessed by phase-contrast microscopy. Cells were plated at

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a density of 2×10^6/35 mm dish on plastic coated with rat-tail tendon collagen (15). The incubation medium was a modified medium 199 (14) prepared with endotoxin-free water (Sigma Chemical Co.) and containing insulin (4 mU/ml), corticosterone (1 μM), penicillin (100 U/ml), and calf serum (5%, vol/vol). After a 4-h period for cell attachment and spreading, serum-free conditions were introduced by changing the medium to one that was identical to the original but with 0.1% (wt/vol) bovine albumin (fraction V; Sigma Chemical Co.) in place of calf serum. Ferric citrate and desferrioxamine mesylate (Ciba-Geigy, Summit, NJ), when present, were added at this time. At 18 h, the medium was changed to one that included ethanol (10 mM) where indicated in individual experiments. Incubation of the cells with ethanol was continued for an additional 6 h, which is sufficient for generating significant levels of chemoattractant in the culture medium (5).

For analysis of cellular iron, hepatocytes were removed from culture plates in phosphate-buffered saline with a Teflon scraper and stored at −20°C in new plastic vials.

Chemotaxis assay. A modification of the leading front method of Zigmond and Hirsch was used, as described previously (5). All buffers were prepared with endotoxin-free water. Human neutrophils from healthy donors were isolated by dextran sedimentation (5). Test fluids (culture media) were assayed routinely at a dilution of 1:200, but other dilutions were assayed in individual experiments to exclude the presence of low-titer chemoattractant or, on the other hand, neutrophil deactivation by a high concentration of chemoattractant (5). Each analysis included negative control media, which consisted of unincubated medium, assay buffer, or medium conditioned by hepatocytes in the absence of ethanol. Results with each of these were not significantly different and constituted background activity. Each assay also included medium containing formyl-methionyl-leucyl-phenylalanine as a positive chemotactic stimulus. A 'checkerboard' analysis was carried out to distinguish true chemotaxis from chemokinetic activity (5).

Biochemical assays. Ethanol metabolism was assayed by the method of Grunnet et al. (16). Culture plates were incubated for 6 h with 2×10^5 dpm [^{14}C]ethanol (10 mM, final concentration) in sealed beakers.

For measurement of iron, cell samples were hydrolyzed in 3.5% nitric acid overnight and analyzed with a flameless atomic absorption spectrometer. The method is sensitive to picogram levels of iron. Heme was measured by the pyridine hemochromogen method with an Amino DW-2 spectrophotometer (17).

Protein was determined by the method of Lowry et al. (18).

Results

Hepatocyte culture: morphology and iron status. Hepatocytes from all animals (iron-deficient, control, and iron-loaded) were readily established in culture, exhibiting ~ 95% viability 24 h after plating on a substratum of collagen-coated plastic. The ultrastructure of the iron-deficient cells was indistinguishable from that of controls, while cells from carbonyl-iron fed rats demonstrated findings typical of iron overload, with ferritin granules in the cytoplasm and in lysosomes (not shown). For all preparations, the total iron content over the period of culture was stable (Table I). Heme was also stable, despite a regular loss of cytochrome P-450 under these culture conditions (15). This may reflect conversion of cytochrome P-450 to its denatured form, cytochrome P-420, rather than complete degradation of the hemoprotein (19).

In hepatocytes from iron-deficient animals, total cellular iron was < 25% of control values after 24 h of culture. In animals receiving chow with added carbonyl iron, hepatocellular iron was markedly elevated (~ 70-fold that of iron-deficient cells), whereas addition of ferric citrate to deficient cells increased the iron levels by only 40–60% or ~ 1 nmol/mg protein (Table I). Iron uptake was unaffected by ethanol (Table II). Addition of desferrioxamine to iron-loaded cells had no effect on the total iron level, possibly because the chelated iron remains within the cell (20).

Because ethanol oxidation is required for chemoattractant production (3), it was important to exclude the possibility that this process was altered in the iron-deficient cells. As shown (Table III), the oxidation of ethanol was at least as rapid in iron-deficient cells as in control cultures.

Chemoattractant production. Chemoattractant was produced by hepatocytes from animals receiving chow or the defined diet with added iron (48 ppm). In both cases, levels were similar to those reported previously (5, 6), and the data are combined as "control" in Fig. 1. By contrast, chemoattractant release by hepatocytes from iron-deficient animals was undetectable, that is, leukocyte migration was not significantly different from that elicited by medium conditioned in the absence of ethanol or unincubated medium. Preincubation of iron-deficient cells with ferric citrate restored chemoattractant production to control levels in a dose-dependent fashion (Figs. 1 and 2), and the time course of the response to ethanol in these repleted cells was the same as that reported previously for normal hepatocytes (5) (Fig. 3). Conversely, pretreatment of iron-loaded cells with the iron chelator, desferrioxamine, ablated chemoattractant production in a concentration-dependent manner (Fig. 1).

Table I. Heme and Iron Content of Rat Hepatocytes at Isolation and after Primary Culture

Type of preparation	Heme iron		Total iron	
	Fresh cells	24-h culture	Fresh cells	24-h culture
	nmol/mg protein			
Control diet (n = 5)	0.19±0.04	0.22±0.04	7.6±0.3	6.3±0.6
Iron-deficient (n = 4)	0.13±0.06	0.18±0.02	1.3±0.1	1.4±0.7*
Iron-deficient + 10 μM ferric citrate (n = 4)	—	—	—	2.3±0.4*
Iron-loaded (n = 3)	0.20±0.50	0.21±0.70	140.0±5.0	135.3±10.0‡
Iron-loaded + 0.5 mM desferrioxamine (n = 3)	—	—	—	129.5±11.3‡
Iron-loaded + 1.0 mM desferrioxamine (n = 3)	—	—	—	139.3 ± 23.2‡

Culture preparation and assay of heme and iron were carried out as described in Methods. The data represent mean±SD. * $P < 0.01$, compared with controls at 24 h; ‡ $P < 0.001$, compared with controls at 24 h.

Table II. Iron Uptake by Iron-deficient Hepatocytes in the Presence or Absence of Ethanol

Type of culture	Hepatocellular iron	
	−Ethanol	+Ethanol
	nmol/mg protein	
Iron deficient	1.7±0.2	1.3±0.3
Iron deficient + 10 μM Ferric citrate	2.3±0.6	2.3±0.8

Hepatocytes were prepared from iron-deficient animals and cultured. The conditions for incubation with ferric citrate and ethanol were as described in Methods. The data represent mean±SD (n = 3).

Discussion

The studies reported here indicate that iron plays a key role in production of the lipid chemoattractant elicited by incubation of hepatocytes with ethanol. Its effect is likely to involve lipid peroxidation, given that iron is a general mediator of this process and that, according to previous studies (9), formation of the chemoattractant involves lipid peroxidation. Moreover, it appears to be a readily accessible ("free") iron pool that is active, as judged by the rapid return of chemoattractant production to iron-deficient cells incubated with ferric citrate and its loss in the presence of an iron chelator. The net change in cellular iron after adding ferric citrate to the culture medium is at most 1 nmol/mg cell protein, which may approximate the size of the iron pool that mediates chemoattractant production. This may be contrasted with a total iron content in control cells of ~ 7 nmol and in the iron-loaded cells of 135 nmol/mg protein. A relatively small size for the "free" iron pool has been inferred also from studies in vitro of liver homogenates, in which < 2% of the total iron was chelatable by desferrioxamine (21).

The findings are in agreement with data indicating that the excess iron in hepatocytes exists as ferritin or hemosiderin (22) and that such storage iron is biologically sequestered (23). Thus, an important controlling factor in the size of the "free" pool may be release of iron from ferritin. While release of iron occurs spontaneously, it is markedly accelerated in the presence of reductants, including superoxide, NADH, and NADPH (24), with concomitant reduction of ferric iron to ferrous. In hepatocytes, ethanol metabolism is accompanied by an increase in the NADH/NAD ratio and also may result in superoxide generation (8). These changes conceivably could accelerate the release of iron from ferritin and expand a pool of reactive ferrous iron.

Table III. Metabolism of Ethanol by Iron-deficient or Control Hepatocytes in Primary Culture

Source of hepatocytes	Ethanol metabolism
	nmol/mg protein/6 h
Control-diet animals	1.3
Iron-deficient animals	2.1

Hepatocytes were incubated in sealed beakers for 6 h with 10 mM [^{14}C]ethanol, as described in Methods. Results are the average of two separate experiments, each determination representing the mean value from six plates of hepatocytes.

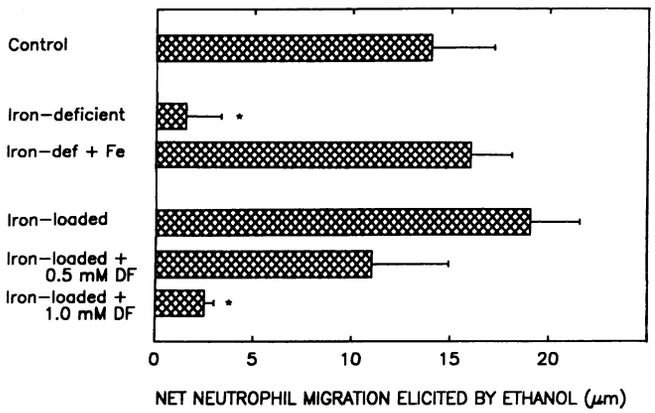

Figure 1. Chemoattractant production by iron-deficient and iron-loaded hepatocytes exposed to ethanol. Hepatocytes were prepared from animal fed iron-deficient or iron-replete diets. The data represent net neutrophil migration as a result of adding ethanol to hepatocyte cultures (see Methods). All culture media were diluted 1:200 before assay. Error bars represent±SD. *P < 0.01 relative to *control*. Iron-def, iron-deficient; Fe, ferric citrate, 10 μM; and DF, desferrioxamine.

The mechanism whereby iron modulates chemoattractant production is currently under study. According to data from a cell-free ethanol-metabolizing system, chemoattractant formation involves oxyradicals (9). The latter apparently are generated by an alternative pathway for acetaldehyde oxidation: acetaldehyde metabolism via xanthine oxidase in vitro yields superoxide. When the reaction is carried out in the presence of arachidonic acid, a chemotactic compound is produced (7) with chromatographic properties similar to those of the chemoattractant produced by intact hepatocytes (F. J. Roll, unpublished data). "Free" ferrous iron may play a central role in this process as an electron donor in the conversion of H_2O_2 to hydroxyl radical (25). The latter oxyradical participates in the oxygenation of lipid to form the chemoattractant (Fig. 4). These findings are in accord with a recent report by Britton et al. showing that an ultrafiltrate of hepatic cytosol of iron-loaded rats can stimulate lipid peroxidation by an NADPH-dependent microsomal system. Inhibition of this effect by low

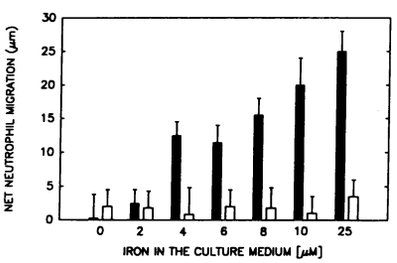

Figure 2. Concentration-dependent effect of iron in restoring chemoattractant production to iron-deficient hepatocytes. Culture preparation was as described in Methods. At 4 h after plating, the indicated concentrations of ferric citrate were introduced. At 18 h, some cultures received medium containing 10 mM ethanol (*filled bars*); ethanol-free controls (*open bars*) were examined in parallel. Media were removed after 6 h of incubation and diluted 1:200 before assay. Error bars indicate±SD (n = 3). *At the highest level of ferric citrate, the indicated chemotactic activity was maximal at a dilution of 1:330, whereas the other samples were diluted 1:200.

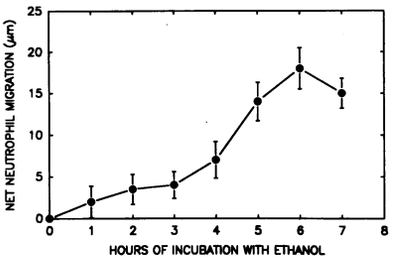

Figure 3. Time course of chemoattractant production by iron-deficient hepatocytes incubated with ferric citrate. Hepatocytes, prepared from iron-deficient animals, were cultured and incubated with ferric citrate (10 μM) and ethanol (10 mM) according to the schedule described in Methods. At each time point, an aliquot of culture medium was removed, diluted 1:200, and assayed for chemotactic activity. Error bars indicate±SD (n = 5).

concentrations of desferrioxamine implicates a pool of low molecular weight iron as the lipid peroxidation catalyst (26). Extrapolation of these concepts to the intact cell implies that iron-generated radicals may escape the endogenous protective systems of the liver. If in persons with alcoholic liver injury the scavenging systems themselves are depleted, the effects of iron-generated radicals may be increased. Indeed, we have recently reported that depletion of glutathione and glutathione peroxidase markedly potentiates generation of chemoattractant activity by ethanol (27). Hepatic glutathione levels, vitamin E and selenium-dependent glutathione peroxidase are acutely depressed experimentally by alcohol (28) and may be chronically depressed on a nutritional basis in alcoholics.

Our studies provide a possible basis for an interaction of iron and ethanol in causing liver injury, an interaction that has been suggested on the basis of clinical observations. The expression of liver disease in hemochromatosis is an example. Although this can occur as a result of marked iron-loading

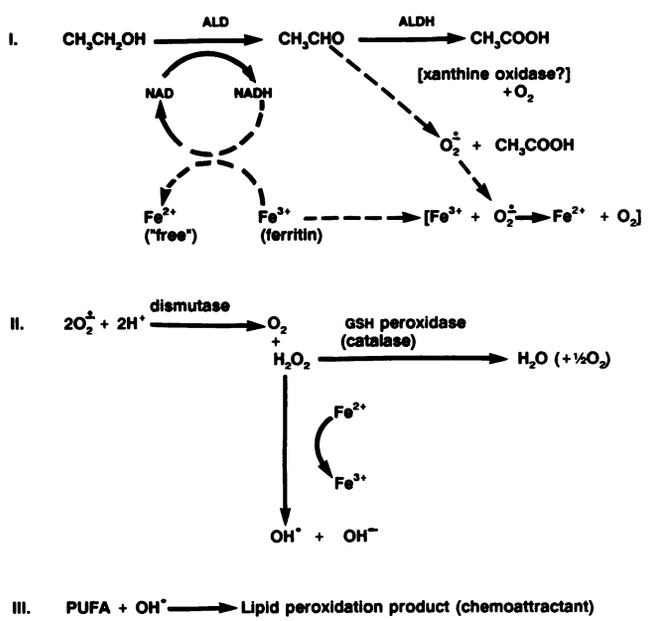

Figure 4. Hypothetical scheme for the role of iron in production of lipid peroxide-derived chemoattractant during ethanol metabolism. Dashed lines (*I.*) indicate alternative pathways of acetaldehyde metabolism leading to production of "free" reduced iron. ALD, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; PUFA, polyunsaturated fatty acid.

alone, it is commonly seen in association with significant ethanol intake, so much so, that in some quarters before the availability of genetic markers of hemochromatosis, the hereditary nature of the disease was questioned (29). In other individuals with alcohol-related liver disease, hepatic iron tends to be increased (30), although at least one report to the contrary exists (31). Although the increase in iron is modest (approximately twofold) and regarded as clinically silent (32), exceptions have been noted. In porphyria cutanea tarda (a disease often precipitated by alcohol abuse), iron clearly plays a central role: iron-depletion therapy brings about a clinical and biochemical response in most, if not all cases, despite the fact that total body iron stores are elevated, on average, only twofold, as in heterozygous hemochromatosis (33). Indeed, evidence exists that some individuals with porphyria cutanea tarda are heterozygous for hemochromatosis (34).

The implication is that individuals in whom iron accumulates (from dietary intake, blood transfusions, or the presence of the hemochromatosis gene) may be predisposed to hepatic injury from alcohol consumption. Both genetic (i.e., the presence of the hemochromatosis gene) and environmental factors may contribute to hepatic iron loading. The prevalence of the gene for hemochromatosis is estimated at 10% of the population (32). On this basis alone, a large number of people are at risk for hepatic iron accumulation. Also, the dietary intake of iron probably is generous, given the practice in the United States and Europe of fortifying flour and breakfast cereal with iron and promoting iron supplements for the general population (35).

Relevant to therapy is the observation that desferrioxamine acutely blocks chemoattractant production, even in iron-loaded hepatocytes. The safety and efficacy of this chelator has been demonstrated in a variety of iron-overload states (36). If the chemoattractant plays a central role in alcohol-related hepatitis, desferrioxamine conceivably could find use as an antiinflammatory agent.

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