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MOLECULAR REGULATION OF IRON HOMEOSTASIS AND RESISTANCE TO INFECTION IN ALCOHOLICS

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1. ABSTRACT

Chronic alcohol abuse is associated with both an altered response to infection and deranged iron homeostasis. While both clinical manifestations are well known, the inter-relationships between alcohol and iron and the response to infection are not. The recent identification of a plethora of iron regulatory and transport proteins has now begun to explain these relationships. This article outlines the current state of knowledge on cellular iron homeostasis, with particular reference to the iron regulatory proteins (IRP1, IRP2 and HFE) and the iron membrane transport proteins, two of which have been shown to be members of the natural resistance- associated macrophage protein family (Nramp1 and 2). Following this introduction, the response of the body to infection, in terms of iron withholding is discussed at the cellular level, especially in terms of the macrophage and its cytokine-mediated responses. Prior alterations to body iron status are also considered in this section. The effect of alcohol alone on the body's response to infection is then outlined, principally in terms of the macrophage function and cytokine regulation. These are then combined to correlate the clinical and experimental observations with known derangements produced by the individual insults of alcohol and altered iron homeostasis. on the response to infection. Particular attention is paid not only to cytokine/chemokine actions, but also to the consequences of the altered production of reactive oxygen and nitrogen species. Finally, the possible mechanisms by which alcohol and altered iron homeostasis lead to tissue damage during infection

2. INTRODUCTION

Chronic alcohol consumption is associated with a spectrum of pathological disturbances to almost every system in the body. Among these, depression of the immune system is now being recognized as one of the more

deleterious consequences of alcohol abuse (1) and may lead to increased susceptibility to infection (2). Patients abusing alcohol also often present with a wide range of clinical manifestations related to disturbed iron homeostasis. There is no one, clear, syndrome, but rather a spectrum of abnormalities, ranging from chronic anemia, through megaloblastic anemia to siderosis and hemochromatosis. Associated with these are alterations in plasma iron turnover, serum transferrin and ferritin, red blood cell iron incorporation and liver iron storage. In over 30% of chronic alcoholics there is a significant increase in liver iron concentrations (3), which is comparable to that seen in treated hemochromatosis patients (4, 5).

Iron and alcohol may be considered independently to be hepatotoxins and both may lead to hepatic fibrosis, cirrhosis and/or hepatocellular carcinoma (6, 7). The pathogenic mechanisms in both cases are similar, involving a combination of toxic liver injury and oxidative stress, leading to the generation of deleterious free radicals (including the hydroxyethyl radical) and/or aldehyde adducts (8, 9, 10, 11, 12). The combination of both of these hepatotoxins is synergistic, leading to collagen deposition (13) and a more rapid pathological liver transformation (14). Liver iron concentrations are considered to be predictive of death in alcoholic liver disease (7). Furthermore, patients with alcoholic cirrhosis have elevated hepatic iron uptake rates (15). In the serum of these alcoholic patients, transferrin (the major serum iron transport protein) turnover is increased (16), and serum ferritin concentrations are elevated (17, 18). (It is still a subject of some controversy as to whether serum ferritin represents an iron scavenger pathway, or merely reflects the consequences of cell death). In small animal models of prolonged alcoholism, however, iron uptake from transferrin is reduced (19), but hepatocyte uptake of serum ferritin iron is markedly elevated (20). These differences between the human and animal studies may be a reflection of the diet used, since it is now known that the Lieber-DeCarli diet used to generate the small animal model of alcohol abuse also results in lowered body iron parameters (21). As the hepatocytes within the liver are not only the major sites for iron storage (in cytosolic ferritin), but also synthesize transferrin, the interaction between iron and these cells probably represents one of the critical points at which alcohol may interfere with iron homeostasis (22).

Under normal circumstances, iron is nearly always “protected” (i.e., maintained in an unreactive form) for most of its transport and storage within the body, and even iron uptake from the gut is tightly regulated. The presence of alcohol, however, perturbs this protection and has been shown to increase the cellular “labile iron pool” (23). [This labile iron pool is normally very small and is thought to represent the cellular transit iron - iron in transit between storage and utilization]. Associated with this elevated labile iron pool is an increase in lipid peroxidation. Both of these can be inhibited by 4-methylpyrazole, an alcohol dehydrogenase inhibitor, but not by alpha-tocopherol, which only prevents lipid peroxidation. While iron and alcohol also have similar effects on hepatic mitochondria, here these two hepatotoxic agents do not appear to have a synergistic mode of action (24). Ethanol is known to increase the iron-dependent production of reactive oxygen species in the mitochondria of alcohol-fed rats, presumably through an outer membrane NADH reductase (25). Although the effects of ethanol on iron have not been examined in relationship to mitochondrial complex I respiratory chain activity, the presence of at least 6 Fe-S sub-units that can be modulated by cellular iron concentrations (26) suggests that this is another area where ethanol may disrupt normal cellular function. Alcohol-related free radicals, possibly produced through the interaction of ferritin-derived iron, have also been implicated in nuclear damage (27, 28, 29). Finally, iron and ethanol have also been implicated in alterations to plasma membrane ATPases (30), at least one of which may be involved in iron transport (see below). Thus ethanol may disrupt iron homeostasis a multiple points on/in the cell, leading to free radical generation, aldehyde adducts, abnormal protein synthesis, oxidative stress, and, ultimately tissue damage.

Where the iron that is displaced by ethanol originates is not known. Originally it was thought that alcohol abuse caused excessive iron absorption, leading to hemochromatosis. It is now known that primary hemochromatosis is caused by a defect in an iron regulatory protein, HFE (see below), and that alcohol probably exacerbates iron uptake in the presence of HFE mutations. Just as serum iron and related parameters vary widely in alcoholics, so do the observations on iron uptake following a prolonged alcohol intake. In the small animal model, using the classical Lieber-DeCarli liquid alcohol diet, hepatocyte iron uptake from transferrin is reduced (19), whereas ferritin iron uptake is markedly increased (20). However, the animals in these and other studies (31) all had lowered hepatic iron parameters (even the controls when compared to normal chow fed animals) and need to be repeated in a model that clearly leads to elevated hepatic ferritin concentrations without the addition of extra iron to the diet (21).

3. NORMAL IRON HOMEOSTASIS

Iron uptake into the body is normally tightly regulated, with only 1-3 mg/day entering the plasma. By contrast, in a normal individual, 35 mg/day is turned over in the plasma, principally derived from senescent erythrocytes. Approximately 85% of plasma iron is transported in the plasma tightly bound to transferrin or ferritin; the remaining iron circulates as low molecular weight complexes and is dialyzable. Four-fifths of this iron is re-utilized in the production of new red cells, while the remaining one-fifth is moved in and out of the hepatocytes according to body requirements. The liver hepatocytes act as major sites of storage for iron, total liver store in cytosolic ferritin being approximately 1-1.5 gm, or 60% of the body total iron stores.

Hepatocellular iron uptake can occur by at least three mechanisms – those involving transferrin binding, ferritin uptake and active transport of non-transferrin-bound iron or low molecular weight iron complexes. It has

Table 1. Intracellular iron regulation

Regulatory Protein	Low iron	high iron
IRP1	Binds to 3' region of TfR mRNA & 5' region of ferritin mRNA – increased TfR and decreased ferritin synthesis	No binding – increased aconitase activity & ferritin synthesis; decreased TfR expression
IRP2	Binds to 3' region of TfR mRNA & 5' region of ferritin mRNA – increased TfR and decreased ferritin synthesis	Increased degradation of IRP2 via ubiquitin-mediated pathway - increased ferritin synthesis & decreased TfR expression
HFE	Not bound to TfR during synthesis –TfR functions normally	Bound to TfR during synthesis and surface expression – TfR function inhibited

generally been considered that, under normal circumstances, the uptake of iron from transferrin represents the major (but not exclusive) pathway of iron into the liver cell. Transferrin (Tf), containing iron, binds to a specific hepatocellular basolateral membrane receptor, TfR, which induces a rapid endocytosis; forming clathrin coated pits and then clathrin coated vesicles or endosomes. (It should be noted that TfR has a much higher affinity for diferric transferrin than apotransferrin at physiological pH). This endocytosis is triggered via tyrosine-based internalization signal located in the N-terminal cytoplasmic portion of TfR (32). Once endocytosed, the pH inside the vesicle rapidly drops below pH 6 due to the membrane proton pumps. At this pH, the ferric iron rapidly dissociates from transferrin and is converted to ferrous iron by a membrane-bound ferrireductase. The dissociation is enhanced by a conformational change in the Tf-TfR complex (33). Unlike most other ligand-receptor complexes, transferrin has a very high affinity for its receptor at pH 5.5-6 and does not dissociate and signal association with a secondary lysosome. The Tf-TfR complex within the endosome is therefore recycled backed to the cell surface. Here apotransferrin has a much lower affinity for TfR than diferric transferrin and is released back into the plasma (34, 35).

Iron uptake via TfR may be altered by regulation of the receptor itself. The discovery and cloning of the HFE gene and isolation of mutations in hemochromatosis has led to a new understanding of iron homeostasis (36). Long known to be associated with chromosome 6, HFE is now known to belong to the MHC class I family and its mRNA may be found in a wide variety of tissues, including liver and intestine (36). Like other MHC class I proteins, HFE associates with $alpha_2$-microglobulin and an absence of $alpha_2$-microglobulin or a defect in HFE results in a failure of the latter to be expressed at the cell surface and consequential iron overload (37,38). HFE also associates with TfR during their biosynthesis and lowers its affinity for transferrin, resulting in decreased iron uptake (39). It has been suggested that failure to bind during their synthesis, prior to translocation, results in lack of iron regulation (40), although this is somewhat controversial (41). Since ethanol is known to disrupt hepatic protein synthesis, it is possible that it interferes with the HFE-TfR production.

The synthesis of TfR is also normally regulated by iron availability through the interaction of two (cytosolic) mRNA-binding iron-regulatory proteins – IRP1 and IRP2. Under conditions of low body iron, these bind to TfR mRNA (which has 5 iron-responsive elements, or IREs) insuring its stability and increasing TfR synthesis. Conversely, under conditions of high body iron, IRP1 is converted to cytosolic aconitase (which contains a 4Fe-4S cluster) and IRP2 is degraded by oxidation and proteasomal degradation (42). However, although IRP2 metabolism is exquisitely responsive to cellular iron concentrations, IRP1 appears to be relatively insensitive (43). The actions of these three regulatory proteins in response to cellular iron may be seen in table 1. This is complicated by the demonstration of a second transferrin receptor – TfR2 (44). TfR2 expression is not regulated by body iron status and thus may contribute to the susceptibility of the liver to iron loading under patho-physiologic circumstances, such as ethanol abuse.

Iron transport across the endosomal membrane can also occur by one of several mechanisms, two of which have been partially characterized. The first requires reduction of ferric iron to ferrous by a membrane–associated ferrireductase (45). The ferrous iron is then transported across the endosomal membrane by a divalent metal ion/proton symport protein. This has been called DMT1 (divalent metal [ion] transporter 1), DCT (divalent cation transporter) or Nramp2 (natural resistance-associated macrophage protein 2) dependent on the organism from which it has been isolated and appears to be tightly conserved from yeasts up to mammalian systems. Nramp1, isolated previously through functional expression cloning, has been shown to be of considerable importance in macrophages to prevent the use of phagosomal iron by invading pathogens (46). Nramp1 appears to have a lower affinity for iron than Nramp2 (which is present on a wide variety of cells, including the liver) and suggests that Nramp1 performs as a bulk iron transport protein for such functions as erythrophagocytosis (47). Nramp2 plays an important role in transmembrane transport of transferrin-bound iron, as may be seen in the Belgrade rat. In this animal there is an Nramp2/DMT1 mutation that leads to defective transferrin processing. Transferrin is internalized by reticulocytes, but the iron fails to be internalized (48). Cytolocalization studies also implicate Nramp2 in this endocytic process in a wide variety of cells (49, 50).

As animal models of iron-deficiency anemia, such as the Belgrade rat still manage to acquire intracellular iron, it is apparent that there are other transport systems present on cell membranes. Recently, Wessling-Resnick and colleagues have isolated a membrane protein,

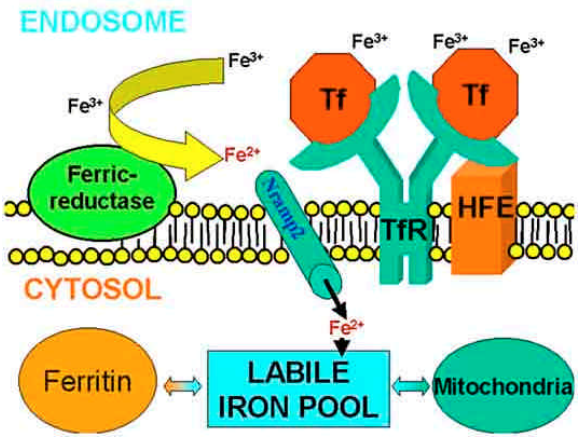

Figure 1. Cellular Iron uptake from transferrin. Ferric iron (Fe^{3+}), bound to transferrin (Tf), is released in the late endosomal compartment, following binding of Tf to its receptor (TfR). TfR binding of Tf is negatively regulated by its membrane association with HFE. Fe^{3+} is converted to ferrous iron (Fe^{2+}) by an endosomal membrane-bound ferricreductase and is then transported into the cytosol via natural resistance-associated macrophage protein 2 (Nramp2), which is a divalent metal ion transporter. Fe^{2+} enters the cytosolic labile iron pool and is then shunted either into storage in cytosolic ferritin, or to the mitochondria.

designated SFT (stimulator of Fe transport) that appears to stimulate the uptake of both transferrin-bound and non-transferrin-bound iron (51). SFT has 6 predicted transmembrane domains and a functionally important RexxE motif that is similar to domains involved in iron transport in yeast and iron binding by ferritin L-chains. This protein stimulates either ferric or ferrous iron at physiologic pH and has been associated with a surface ferrireductase activity (52). There is also a low affinity ferrous iron uptake system that is unimpaired in Belgrade rats (53) and a ceruloplasmin-stimulated ferric iron transport system (54), which have yet to be fully characterized.

While high affinity, energy-dependent mechanisms for non-transferrin-bound iron have been described for liver cells (55, 56, 57, 58, 59), none of the transport proteins have been defined and compared to those above. At least one mechanism, however, has been suggested to involve the generation of hydroxyl radicals (60). Although ferritin and lactoferrin are also endocytosed (61, 62, 63) it is not known with any certainty whether this mechanism involves specific protein-receptor-mediation, although one group has claimed to have isolated a specific ferritin receptor (64), or whether ferritin or lactoferrin bind to other receptors such as the asialoglycoprotein receptor (63). Since serum ferritin is elevated following alcohol abuse (see above) and lactoferrin levels increase markedly following infection (a not uncommon event in alcoholics), iron derived from both of these sources could also contribute to the non-transferrin-derive route of cellular iron uptake and so result in changes to the labile iron pool.

Once transported across the cell membrane, iron enters the cytosol and becomes part of the labile iron pool. Under normal circumstances, the concentration of labile iron is of the order of 0.25-2 micromolar, depending on the cell (65, 66, 67), although hepatocytes have been variously reported to have a range of 3-12 micromolar (68, 69). Iron from this compartment is rapidly transferred to either mitochondria, or into storage in cytosolic ferritin. It is within this compartment that, because of the synthesis of the transferrin receptor, ferritin and the various iron transport proteins that most regulation and perturbations of iron homeostasis probably occur. A diagrammatic outline of iron transport across the endosomal membrane may be seen in figure 1. This labile iron pool is clearly dynamic, as has been demonstrated by exposure of K562 erythroleukemia cells to ferrous salts or oxidative stress. Here the iron pool expanded from 350 nM to 420 nM, but the return to normal values varied between 35 and >90 minutes, dependent on the treatment (66). Oxidative stress also significantly stimulated ferritin synthesis (65). Intracellular labile iron concentrations are considered by some researchers to reflect the cell's susceptibility to oxidative stress (70). There is no doubt, however, that the cytosolic iron pool does regulate iron uptake, principally through changes in IRP2. Under conditions of low body iron, this protein, like IRP1, binds to TfR mRNA (which has 5 iron-responsive elements, or IREs), insuring its stability and increasing TfR synthesis, and to ferritin mRNA, which has a single IRE in its 5' UTR, blocking the binding of 43S pre-initiation complex to the mRNA and repressing ferritin synthesis (71, 72, 73). In response to high cellular iron, IRP2 is degraded by oxidation and proteasomal degradation (42). IRP2 regulation is thus very responsive to cellular iron concentrations, whereas IRP1 is more sensitive to oxidative stress (74). Since ethanol induces increases in the labile iron pool and iron-mediated lipid peroxidation in liver cells (23), it is conceivable that ethanol disrupts iron regulation by both of these IRP's. Alternatively, the increase in the labile iron pool and lipid peroxidation may be caused by iron release from cytosolic ferritin and mediated by cytochrome P4502E1 (75).

Iron transport into mitochondria is an important but often overlooked step in cellular iron homeostasis. Mitochondria in fact utilize most of the cellular iron to produce heme- and iron-sulfur cluster-containing proteins, such as the cytochromes and (mitochondrial) aconitase. Various researchers have in fact observed that (a) iron overload induced mitochondrial dysfunction, with associated lipid peroxidation and decreased mitochondrial ATP content (76), (b) that ethanol abuse was associated with increased reactive oxygen species production by the mitochondria (25), and (c) that iron and alcohol failed to have a synergistic effect on either mitochondrial lipid peroxidation or oxidative metabolism (24). One of the possible mechanisms leading to mitochondrial iron overload may involve the frataxin molecule. The synthesis of this mitochondrial protein is defective in Friedreich ataxia and is associated with increased myocardial iron deposition and defective mitochondrial respiration (77). The yeast homologue is now known to mediate mitochondrial iron efflux (78) and deletion of the gene

increases the mRNA of several yeast IRP's and results in a significant increase in mitochondrial iron content (79).

Relative to iron uptake, very little is known about iron efflux from cells. For many years, ceruloplasmin was considered to play a role, but this is now thought to be minor (80). Heme oxygenase-1, a heat-shock protein is now considered to be important and is thought to have a role in iron recycling through its facilitation of the release of heme-iron from hepatic cells, although the mechanism is unknown at this time (81, 82). In some cells, such as macrophages, Nramp1 is thought to function as a divalent metal efflux pump at the phagosomal membrane and probably facilitates iron release from these macrophages during erythrophagocytosis (83, 84). Finally, another iron transporter, Ireg1, or ferroportin1, has recently been cloned, localized to the basolateral membrane of cells, and characterized as being involved in iron efflux (85, 86). The mRNA for this iron exporter has a typical IRE in the 5' UTR, suggesting that Ireg1 is down regulated under conditions of low iron, and up-regulation has been observed during hypoxia (86). Although it does not appear to change rapidly, its activity does diminish with an increase in hematocrit.

4. IRON AND RESISTANCE TO INFECTION

There is a continuous battle for iron between invading pathogens and the host organism. Most invaders require iron for proliferation and the host organism responds by attempting to withhold unprotected iron (mainly in the plasma) from them. Iron withholding results in hypoferremia and/or the anemia of inflammation. It is generally considered that the hypoferremia/anemia results from a blockade of the macrophage release of senescent RBC-derived iron (87, 88). However, there are also several other mechanisms that cause the lowering of the plasma iron concentrations. Liver parenchymal cells respond by increasing the uptake of transferrin-bound iron and its subsequent storage (89). Unpublished data suggest that this is a cytokine-mediated response. Another method of sequestering iron is via the secretion of lactoferrin. Iron-free lactoferrin (apolactoferrin) is thought to be secreted by phagocytic cells at the site of inflammation as a result of either interleukin 1 or complement C5a stimulation (90). Apolactoferrin has been shown to avidly acquire iron from transferrin at acid pH, which is often found in inflamed areas (91). Although lactoferrin levels are only raised approximately three times above its normal microscopic concentration during infection (92), its effect maybe much greater than anticipated because of its biological half-life is so short and iron-loaded lactoferrin is rapidly removed from the plasma by either mononuclear phagocytes or liver cells.

The problem with iron withholding by the host organism is that it is also required for the host defense mechanisms. Several groups have shown that iron modulates cytokine activities, nitric oxide production and even immune cell proliferation (93). Thus, it is essential that iron be effectively unavailable to the invading pathogen, but, at the same time, immediately available for mounting a successful immunological defense (94). Both iron overload and iron deficiency appear to be associated with an increased risk of infection. Central to this conundrum appear to be the Nramp proteins and regulation of the labile iron pool. The Nramp protein family plays a key role in iron homeostasis and has been tightly conserved from unicellular organisms through to the higher primates (95, 84). It is interesting to note that bacterial virulence is associated with the genes that code for iron acquisition(96, 97). Nramp polymorphism in man has also been correlated with disease susceptibility in a variety of diseases (98, 99, 100). Nramp1 has been shown to regulate both intracellular pathogen proliferation and the macrophage inflammatory response. Conversely this protein has also been shown to be induced by inflammatory stimuli (101). Although it is well known that Nramp1 transports iron and other divalent metal ion across membranes, the direction is somewhat controversial; indeed it may be that it is bi-directional and transport ions against a proton gradient (102, 103). While Jabado et al. (104) have shown that Nramp1 transports Mn^{2+} from the phagosome into the cytosol others have shown that it transports iron from the cytosol into the phagosome and contributes to intracellular killing through the generation of hydroxyl radicals (105, 106). Nramp1 is also involved in the regulation of phagosomal pH, although the mechanism is unclear and may involve recruitment of H^+-ATPase activity (107) or mediation of microtubule dependent phagosomal and/or lysosomal transport (108). It has also been suggested that protein kinase C modulates Nramp1 activity through phosphorylation (109), probably in the amino terminal domain (46). It remains to be determined whether there is a negative feedback loop between protein kinase C activity and Nramp1 function. This becomes more complicated because sit has been proposed that nitric oxide may be involved in these regulatory pathways (110).

Unlike Nramp1, which is only expressed in macrophages and a few other tissues (such as the liver), Nramp2 has been detected in most tissues (111). As has been mentioned above, Nramp2 is now known to be involved in the transport of ferrous iron is then transported across the endosomal membrane and is a divalent metal ion/proton symport protein. Although both proteins are upregulated following bacterial infection, it is now thought that Nramp2-related host defense function is unrelated to that of Nramp1 and that it can be regulated independently of the known macrophage IRP's (112). Although both interferon-gamma and endotoxin (LPS) increase both Nramp2 and IRP1, paradoxically, they decrease transferrin receptor mRNA expression in macrophages (113). Thus these activated macrophages would increase their uptake of low molecular weight iron complexes, while reducing their interaction with transferrin – at least partially explaining the iron-withholding phenomenon seen following infection. It is also known that Nramp1 is upregulated by both interferon-gamma and LPS; the response elements being found in the 5'regulatory region of Nramp1 (114).

Since macrophages are stimulated by microbial degradation products, such as LPS and cytokines released as a result of inflammation and infection, any iron regulatory protein that can respond to these will alter

cellular iron homeostasis. Since it is now well known that many of the cytostatic and cytotoxic properties of macrophages elicited during infection are produced by nitric oxide generation, the fact that both IRP1 and IRP2 interact with this molecule is of some interest. It has now been shown that $NO^{\cdot}$, which interacts primarily with iron, activates IRP1 binding activity, leading to increased transferrin receptor mRNA concentrations. However, NO^{+}, which nitrosolates thiol groups, reduces the mRNA binding of IRP2 and leads to its degradation, results in a decrease in transferrin receptor mRNA levels (115). A similar effect is seen following treatment with interferon-gamma and LPS and can be prevented by treatment with inducible nitric oxide synthase (iNOS) inhibitors (116). This is also accompanied by an increase in cellular ferritin synthesis. It has also been suggested that the induced nitric oxide production results in increased IRP1 activity in adjacent cells (117). It should also be noted that changes in oxygen tension regulate both IRP binding activities and thus reactive oxygen species will also have an effect on cellular iron regulation (118). IRP1 has been shown to be activated by oxidative stress (74). Conversely, hypoxia has been shown to post-translationally activate IRP2 (119). Heme oxygenase, which is considered by some workers to be IRP2, has also been shown to be induced and confer some protection in a kidney model of acute oxidative stress involving LPS administration interferon-gamma and (120). This was also accompanied by increased cellular ferritin synthesis.

Inflammatory cytokines both alter iron homeostasis not only by direct interaction with the regulatory and transport proteins but also by the activation of transcription factors such as NF-kappaB. Not only is there endothelial cell blockade, but also enhanced liver parenchymal cell uptake of transferrin bound iron in response to cytokine stimulation (121) and not to direct stimulation of the hepatocytes by LPS (89). In primary idiopathic (hereditary) hemochromatosis, tumor necrosis factor alpha polymorphism has been shown to correlate with ALT values in individuals with the C282Y mutation and also with siderosis, suggesting that TNF alpha may modulate the severity of liver damage associated with iron overload in this disease (122). The effect may be an exacerbation of defective iron homeostasis as a result of the HFE mutations, especially since HFE belongs to the MHC class I family. However, in experimental animals, deletion of this molecule, while leading to iron overload does not produce obvious alterations to the immune system (123). However, in patients with HFE gene mutations who contract viral hepatitis iron overload was more pronounced (124). In hepatitis C infection, iron overload also appears to be viral subtype-specific (125). TNF alpha and NF-kappa B appear to be causally related in chronic liver diseases, particularly that caused by alcohol abuse (see below) and this effect also appears to cause a modest increase in non-heme iron deposition in Kupffer cells (126).

5. ALCOHOL AND INFECTION

Chronic alcohol consumption is associated with a wide variety of pathological manifestations, including suppression of the immune system, leading to increased morbidity and mortality. However, whether this clinical observation (127) is due to alcohol, liver disease or concomitant malnutrition in human alcoholics is unclear (128). Studies with animal models, however, have shown that ethanol not only modifies the immune response, but also actually enhances susceptibility to infectious pathogens. In particular, ethanol has been shown to significantly alter cytokine responses (129). While antibody production to T-cell antigens is affected by alcohol consumption, T-cell-independent antibody production is normal in animal models of alcohol abuse (130), leading to the hypothesis that ethanol primarily inhibits T-cell function.

However, more recent work suggests that ethanol also markedly affects monocyte/macrophage function (131). Acute ethanol administration to mice significantly decreases TNF alpha, IL-1 beta, and IL-6 production in both alveolar and peritoneal macrophages following a bacterial challenge. The decreased antigen-specific T-cell proliferation has been shown to be related to decreased monocyte antigen presentation (132). This inhibition of monocyte TNF alpha production is, partially mediated through the increased production of IL-10 -at least *in vitro* (133). While these effects are transient, they last for at least twenty-four hours. These findings imply that even acute ethanol intoxication is likely to impact host defenses and therefore continued alcohol abuse may alter their long-term capability to mount a longer-term immune defense. Furthermore, such a dampened immune response will lead to more rapid progression in such diseases as hepatitis C infection, where inflammatory mediators have an impact on the clinical outcome.

6. ALCOHOL AND IRON IN THE CELLULAR RESPONSE TO INFECTION

One of the more recent findings has been the association of hepatic iron deposition, frequently associated with moderate-to-heavy alcohol abuse, in subjects with hepatitis C infection and the associated resistance to "conventional" interferon therapy (134, 135, 136). While this observed iron deposition is primarily parenchymal, it is clearly a manifestation of disturbed iron homeostasis in these patients. Furthermore, these findings also correlate with increased liver damage and more rapid progression of the disease. A number of studies have that the HCV core protein plays a role in the virus induced pathogenesis, which includes suppression of the host immune response, and that this, combined with associated factors such as alcohol abuse contribute to the perturbations to iron homeostasis. This, in turn results in the increased generation of free radicals and NF-kappa B activation leading to an increased production of cytokines and chemokines, such as RANTES, MCP-1 and MIP-1 (12), and a more rapid disease progression.

Alcohol and iron have both been shown independently to be hepatotoxins and to cause progressive liver damage. While some of the effects may be related to progressive iron loading of the parenchymal cells and the

Table 2. The effects of ethanol on hepatic macrophages[1]

UPREGULATION	DOWNREGULATION
Free radical generation	Phagocytosis
NF-kappa B activation	Microbial killing
Adhesion molecules	Antigen presentation
Secretion of cytokines & chemokines	FcR & mannose-specific receptors
Protease	MHC II

[1] Modified from 141.

production of highly toxic free radicals (such as the hydroxyl radical), both alcohol and iron also influence the inflammatory response in macrophages, especially the hepatic macrophages (Kupffer cells). Table 2 shows a summary of the various effects of ethanol on macrophage function, as typified by the Kupffer cell:

As can be seen from this table, ethanol has both upregulatory and downregulatory effects on macrophage functions. For example it stimulates the secretion of cytotoxic mediators, which may contribute to hepatic injury. On the other hand, it also has a definite immunosuppressive effect in terms of macrophage microbicidal functions. Thus, alcohol may exacerbate a disease process by reducing the host defenses, leading to a prolonged or worsened infection, while, at the same time, producing proinflammatory modulators that affect other cells such as hepatocytes and endothelial cells.

It is now generally considered that alcoholic liver injury results from activation of hepatic macrophages by endotoxin, due to increased translocation from the gut into the portal circulation (137). However alcoholics are also more susceptible to infections of all types, which could also add endotoxin to the circulation. During infection or sepsis, iron is either withheld from the circulation in the liver and splenic macrophages or re-routed to the liver parenchymal cells for storage. In consequence there is a significant, if transient, increase in the labile iron pools of these cells. As a result, this increased non-protected iron pool may react with cellular hydrogen peroxide to form more toxic radicals such as the hydroxyl radical. Pre-existing increased iron concentrations in these cells due to pathological conditions such as idiopathic hemochromatosis, sickle cell anemia, treated thalassemia, and late stage alcoholism may further compromise the host through similar mechanisms during infection.

The increase in liver iron deposits, particularly in the parenchymal cells may occur as a result of alcohol-induced endotoxemia. Unpublished data from our laboratory has shown that both recombinant TNF alpha and LPS from E. coli enhanced transferrin-bound iron uptake by hepatocytes from both normal and alcohol-fed rats, reversing the depression of iron uptake normally seen following alcohol alone (19). It should be noted that iron deposition in the hepatocytes from alcohol-fed rats was also significantly reduced. This may reflect the effects of the classic liquid diet used in these studies since later work using the agar block technique of alcohol feeding actually significantly increased the non-heme iron concentrations after 8 weeks to almost double that of the control animals (21)! While no liver damage, apart from mild steatosis is observed with these models, the addition of either saturated fat to the agar blocks or iron to the liquid diets administered by intragastic catheter leads to much more marked liver injury (138). Addition of endotoxin and ethanol also leads to an increase in the uptake of ferritin-bound iron by both hepatocytes and Kupffer cells (139). Because of this, it appears that iron could also be involved in increasing the sensitivity of hepatocytes to the cytotoxic action of beta-chemokines such as MIP-2 during alcohol abuse (140). That iron is involved in this sensitization can also be deduced through the prior administration of iron-dextran to alcohol fed rats where treatment with recombinant MIP-2 significantly increases its cytotoxic effects (141). It is also interesting to note that desferrioxamine reverses the hepatocyte dysfunction and associated mortality in a rat model of sepsis, irrespective of their prior body iron status (142, 143).

One of the key mediators in the pathogenesis of tissue injury is TNF alpha. Amongst its many actions is that of the stimulation of both oxidative stress and the upregulation of other cytokines, chemokines and adhesion molecules. TNF and endotoxin have been implicated in several models of alcohol-mediated liver injury. The classical liquid alcohol diets have been shown sensitize animals injected with endotoxin to produce significantly more TNF as well as inducing greater hepatotoxicity (144, 145). Nanji's group has not only shown that LPS administration to rats fed alcohol intragastrically results in increased TNF mRNA, but that this production coincided with the development of liver injury (146). The liver injury can be reduced in a variety of ways, such as gut sterilization or feeding lactobacillus (to reduce endotoxemia), gadolinium chloride administration (to destroy the Kupffer cells) or cimetidine treatment (to reduce the generation of ROS via P4502E1) (147, 148, 149, 150). Although cells such as hepatocytes are normally protected from TNF cytotoxicity by a variety of agents, including nitric oxide and the synthesis of acute phase reactants, it is thought that sensitized hepatocytes fail to make these agents (151) and this subsequently leads to cytokine-induced liver damage.

One of the more important pathways involved in the generation of both TNF and nitric oxide is that involving the redox-sensitive transcription factor NF-kappa B. It also appears that early release of nitric oxide upregulates NF-kappaB, while later release downregulates it (152). TNF binding to its cellular membrane receptor can lead to one of two alternatives: the NF-kappa B survival route, or the "death pathway, as may occur with alcohol-enhanced acetaminophen hepatotoxicity (151). A similar effect has been seen by these workers with the administration of a proteosome inhibitor (MG132) to HepG2 cells; these cells are then susceptible to TNF-mediated apoptosis.

The mechanisms involved in the development of alcoholic liver disease are many and both oxidative stress and iron have been implicated in its pathogenesis. Chronic alcohol consumption leads to mild siderosis in

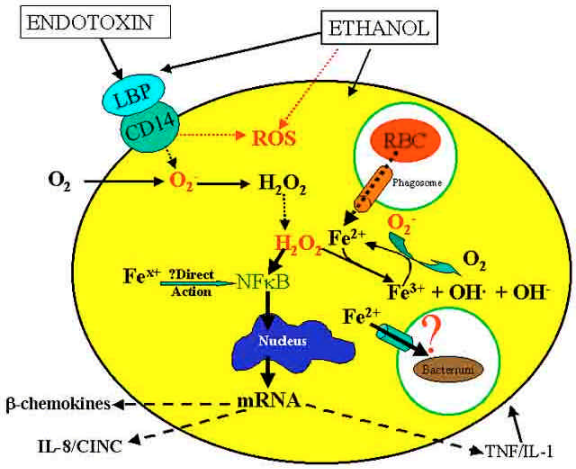

Figure 2. Inter-relationships between alcohol and iron in macrophages during infection

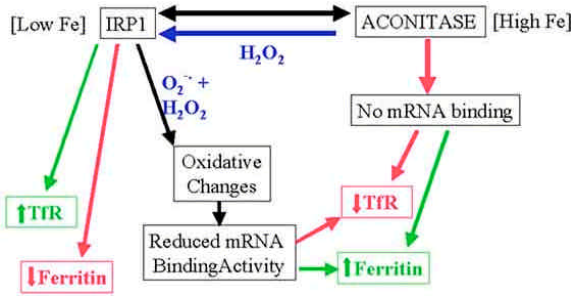

Figure 3. Normal and oxidative-stress-related pathways of IRP1-regulated iron homeostasis. Under conditions of high intracellular iron concentrations, IRP1 exists as cytosolic aconitase and does not bind to TfR or ferritin mRNAs. This leads to decreased TfR expression and increased cytosolic ferritin synthesis. The presence of hydrogen peroxide, however, will result in an increase in IRP1 activity, *irrespective* of the cellular iron concentration, leading initially to increased transferrin-bound iron uptake and decreased ferritin synthesis. Prolonged exposure to superoxide or ROS and hydrogen peroxide, however, causes oxidative changes to IRP1 structure, leading to reduced mRNA binding and subsequently lowered levels of TfR and increased synthesis of ferritin.

approximately one-third of alcoholic cirrhosis patients (see 153, for a review) and this may be shown to occur experimentally, although it depends on the (small) animal model used (154,21). Conversely experimental iron supplementation can also result in increased liver injury (155, 154). Although the increased non-heme iron in the cells was only modestly above control values, and stainable iron measurement was indistinguishable between the alcohol-fed and control groups, nevertheless liver damage was significant in the iron and alcohol animals. Thus it would appear that the two insults act at the very least additively and at the most synergistically to produce liver damage. It is probable that two processes could be occurring: (1) increased shunting of iron into the labile iron pool or movement away from (protected) ferritin storage, and/or (2) increased activation of NF-kappa B. Thes steps are shown in figure 2. Because of the difficulties associated with measurements of the labile iron pool, these do not appear to have been performed in these models at the present time. However, very preliminary data generated in our laboratory suggest that, at least in HepG2 cells, H-ferritin mRNA and ferritin protein synthesis are increased over 7 days, along with a progressive increase in the labile iron pool concentration, in response to alcohol alone in the medium. It remains to be ascertained which of the many steps or modulators outlined above have been altered by alcohol and whether the effect is due to the direct action of alcohol metabolites or some intermediate secondary step.

While the mechanism(s) for altered iron homeostasis in response to alcohol and infection remain(s) to be characterized, some research has been carried out on iron's action in generating tissue injury by pathways other than free radical generation. Tsukamoto and colleagues (156) have shown that iron mediated potentiation of alcoholic liver injury is associated with increased NF-kappa B activation, upregulation of NF-kappa B-responsive chemokine gene expression and mononuclear cell infiltration. Further work by this group (138) has shown that increased NF-kappa B activation is associated with elevated iron stores and that treatment with a lipophilic iron chelator (deferiprone or L1) normalized both of these *ex vivo*. Furthermore, erythrophagocytosis by Kupffer cells isolated from these alcohol-fed animals both increased cellular iron concentrations (as would be expected from other studies) and accentuated NF-kappa B activation. Both effects could be ameliorated by pretreatment of the cells with a heme oxygenase inhibitor (zinc protoporphyrin). However, while they consider that heme-derived iron plays a pivotal role in priming macrophages for NF-kappa B activation, it should also be noted that heme oxygenase 1 is also involved in iron efflux regulation and is considered by some "ferricologists" to be an intracellular iron regulatory protein. Nonetheless, these researchers have begun to unravel the link(s) between iron, alcohol and infection.

Oxidative stress, resulting from iron-derived free radicals, plus those derived from alcohol and its metabolism will clearly also alter intracellular iron homeostasis. Production of hydrogen peroxide or ROS is known to cause a reversible loss of aconitase enzyme activity without increasing IRP1 binding activity. They can also alter IRP1 binding activity, resulting in an increase in ferritin synthesis and a decrease in the number of transferrin receptors, as may be seen in figure 3.

On the other hand, inflammatory cytokines cause activation of NO pathways. These will differentially activate IRP1 and IRP2, leading to induction of IRP1 binding activity, but IRP2 degradation! Thus, theoretically, it is possible for NO to both upregulate and downregulate intracellular iron homeostasis.

7. CURRENT PERSPECTIVES

It is clear that ethanol interferes both with intracellular iron homeostasis (particularly in the liver) and

the immune responses to infection. The altered iron homeostasis in turn also modulates the immune response, and both alcohol and iron increase the cellular production of free radicals leading to oxidative stress and exacerbated tissue damage. What has still to be ascertained is the just where ethanol interferes with iron homeostasis. While it is now apparent that one of the manifestations of alcohol intoxication is an altered labile iron pool, it is still unclear how this occurs. Since some of the iron transport proteins are also associated with the macrophage response to invading bacteria (the Nramp proteins) and others with the MHC class I family of proteins (HFE), it is clear that iron fluxes are intimately involved in the host defense against invading pathogens. Thus derangement of iron homeostasis by ethanol ultimately leads to derangements in the immune response. Further research is required therefore, to enhance our understanding of the detailed regulation of intracellular iron homeostasis, its role in the immune response and just how alcohol deranges this tightly controlled system.

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