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EFFECTS OF ACUTE ETHANOL ADMINISTRATION ON THE UPTAKE OF ^{59}Fe-LABELED TRANSFERRIN BY RAT LIVER AND CEREBELLUM

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Abstract—The uptake of iron by the liver and cerebellum was measured in rats using [^{59}Fe]transferrin. An acute ethanol load (50 mmol/kg body wt., i.p.) elicited a significant increase in the hepatic and cerebellar non-heme iron concentration. The uptake of ^{59}Fe by the liver and the cerebellum was significantly greater in the ethanol-treated rats than in control animals. The administration of allopurinol prior to the ethanol load prevented the changes in liver and cerebellar non-heme iron content. Moreover pretreatment with allopurinol reduced the ethanol-induced enhancement of ^{59}Fe uptake by the liver and completely prevented the changes in ^{59}Fe uptake by the cerebellum. These effects of allopurinol lead us to suggest that oxygen-derived free radicals are involved in the ethanol-induced disturbances of iron uptake both at the hepatic and cerebellar level.

Key words: ethanol; allopurinol; transferrin–iron; iron uptake; liver; cerebellum

Hepatic iron overload is commonly observed in about 30% of alcoholics [1]. Alcohol intake appears to alter iron metabolism, predisposing to excess iron storage and possibly iron damage. In rodents, chronic ethanol feeding has also been reported to increase the liver iron content [2, 3]. We have reported previously that not only long-term ethanol administration but also an acute ethanol load elicits an increase in hepatic iron [4, 5]. We have also shown that such an effect of ethanol is not restricted to the liver but is also observed in the cerebellum [5]. The increase in the non-heme iron content was accompanied by an enhancement in the low molecular-weight non-heme iron compounds (LMW-Fe†) content both in liver and cerebellum [4, 5]. Since LMW-Fe plays a central role in the catalysis of free radical-mediated reactions, we have suggested that the increase in LMW-Fe, which, in turn, can stimulate the formation of injurious radicals, is an important factor contributing to ethanol-induced cell damage [5].

The mechanisms behind liver and cerebellar iron accumulation associated with ethanol intake are still obscure. Among the mechanisms by which ethanol could increase iron content of the tissue, a direct pharmacological effect of ethanol, such as increased hepatic and cerebellar iron uptake from transferrin, can be proposed. During studies concerned with the effect of ethanol on iron uptake by isolated rat hepatocytes conflicting results, which may be related to differences in experimental conditions, have been reported [6–8].

As part of an ongoing investigation into the effects of ethanol on iron metabolism, we studied the *in vivo* effects of an acute ethanol load on iron uptake from [^{59}Fe]transferrin by rat liver and cerebellum. Having previously observed that allopurinol, a xanthine oxidase inhibitor, prevents changes in non-heme iron and affords protection against the oxidative stress induced by ethanol at the hepatic and cerebellar levels [9, 10], we also studied the effects of allopurinol on the ethanol-induced changes in iron uptake.

MATERIALS AND METHODS

Chemicals. The radioisotope ^{59}Fe (as $^{59}FeCl_3$ in 0.1 M HCl, 3–20 mCi/mg iron) was purchased from Amersham International (France). Human transferrin (98% stated purity) and all other reagents (analytical grade) were from the Sigma Chemical Co. (St Louis, MO, U.S.A.)

Labeling of transferrin. Apotransferrin was prepared by incubating diferric transferrin in 30 mM EDTA/0.6 M sodium acetate buffer (pH 5), then dialysed against 0.1 M. $NaClO_4$/0.02 M sodium bicarbonate buffer (pH 8.5) (24 hr, two changes, 4°) the final solution being adjusted to 0.6 M $NaHCO_3$ and subsequently labeled with ^{59}Fe. The methods for this labeling have been described previously [11, 12]. Briefly, ferric nitriloacetate was prepared by adding equal volumes of nitriloacetic acid (NTA) and ferric chloride ($^{59}FeCl_3$) (molar ratio 2:1) in 10 mM Tris buffer (pH 7.1). Subsequently, 0.8 equivalent of ferric nitriloacetate [$^{59}Fe(NTA)_2$] was added to the apotransferrin at room temperature while gently stirring for 30 min. Unbound ^{59}Fe and free NTA were separated from the protein by gel filtration. The [^{59}Fe]transferrin was applied on a G-

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† Abbreviations: LMW-Fe, low molecular-weight non-heme iron compounds; PMN, polymorphonuclear leucocytes.

25 sephadex gel column equilibrated and eluted with 0.1 M $NaClO_4$ and then the eluted [^{59}Fe]transferrin fraction was applied on a G-25 sephadex gel column equilibrated and eluted with NaCl 0.9%. The final iron saturation of transferrin was about 40%.

Animal experiments. Male Sprague–Dawley rats about 280 g in weight and fed a standard pellet diet (Iffa Credo, UAR) containing 100 ppm iron were used. Ethanol (50 mmol/kg body weight, i.p.) was administered as a 20% (v/v) aqueous solution to overnight fasted animals. Allopurinol (146 μmol/kg, i.p.) was administered 16 hr and 20 min prior to the ethanol load. Control animals were given the same volume of saline. [^{59}Fe]Transferrin was injected 10 min after the ethanol load via the penis vein, under light diethyl ether anesthesia. Each animal received 1 μg of labeled protein/g of body weight in a total volume of 0.4 mL.

An initial blood sample was collected by cardiac puncture 5 min after the injection of labeled protein and further blood samples were taken by cardiac puncture at 30, 60 or 90 min. Following the removal of the last blood sample, the animals were immediately decapitated and the liver and the cerebellum rapidly removed. Blood samples were centrifuged, the plasma removed and analysed for non-heme iron content and radioactivity. The cerebellum and a sample of the liver were washed, mopped up and used for analysis of the non-heme iron content and of the radioactivity. Weighed samples of cerebellum (0.1 g) and liver (0.2–0.3 g) were homogenized in 2 and 5 mL 0.15 M NaCl, respectively, and were analysed for radioactivity using a Packard COBRA gamma counter.

The amount of radioactivity attributable to plasma contamination was subtracted from the hepatic and cerebellar ^{59}Fe values. For this purpose, we determined the hematocrit value in the final whole blood sample and the haemoglobin content of the tissular samples. Plasma iron and tissue non-heme iron were determined by a method described previously [5] which is characterized by a sensitivity of 3 ng/mL and a variation coefficient < 5%. Blood to cerebellum transport rate measured in mL/g-min (K_{in}) was determined by a graphical method as described by Patlack *et al.* [13] and applied by Blasberg *et al.* [14].

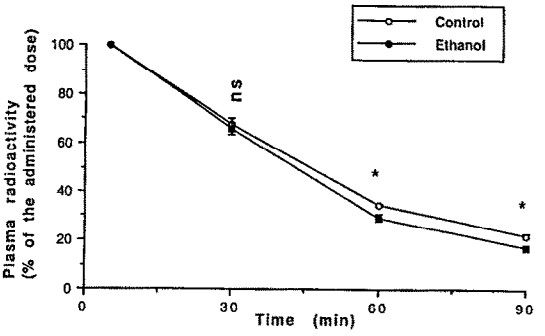

Fig. 1. Effect of an acute ethanol load on the disappearance of plasma ^{59}Fe. Overnight fasted rats received ethanol (50 mmol/kg, i.p.) 10 min before the label. At zero time [^{59}Fe]transferrin was administered intravenously. Results are expressed as percentages of the respective values measured in a sample taken 5 min after injection. Points represent the means from 5–9 animals, the bars are standard errors of the means. *Indicates significantly different from control group at $P < 0.01$.

Statistical interpretation. Data are expressed as means ± SEM. ANOVA or Student's *t*-test were used to assess significance.

RESULTS

Iron status of the rats. Following the acute ethanol administration (50 mmol/kg body wt, i.p.), the mean hematocrit value did not differ from that of the controls (41.8 ± 2.4 vs 42.5 ± 1.4, $P > 0.05$). Plasma iron dropped significantly (by 18 and 28% at 70 and 100 min, respectively), after the ethanol load. Conversely, the hepatic and cerebellar total non-heme iron contents increased, significant changes ($P < 0.001$) being achieved 100 min after the ethanol load (Table 1).

Iron uptake by the liver and the cerebellum. As shown in Fig. 1, the plasma concentration of ^{59}Fe

Table 1. Non-heme iron levels in plasma, liver and cerebellum after an acute ethanol load

Time (min)	40	70	100
Plasma			
Control	13.2 ± 0.6 (4)	13.3 ± 0.7 (4)	13.4 ± 0.5 (7)
Ethanol	13.1 ± 0.3 (8)	11.2 ± 0.4 (8)†	9.6 ± 0.5 (7)†
Liver			
Control	1.96 ± 0.11 (4)	1.88 ± 0.19 (4)	2.02 ± 0.10 (7)
Ethanol	2.02 ± 0.11 (8)	2.07 ± 0.10 (8)	2.30 ± 0.09 (7)†
Cerebellum			
Control	0.121 ± 0.006 (4)	0.113 ± 0.007 (4)	0.126 ± 0.006 (7)
Ethanol	0.121 ± 0.007 (8)	0.132 ± 0.009 (8)*	0.170 ± 0.005 (7)†

Ethanol (50 mmol/kg) was injected i.p. to overnight fasted rats. Values are expressed as μmol/L plasma or μmol/g liver or cerebellum (wet wt). Values are means ± SE. Number of rats in parentheses.

Statistical significance: *$0.02 < P < 0.05$; †$P < 0.001$ vs controls.

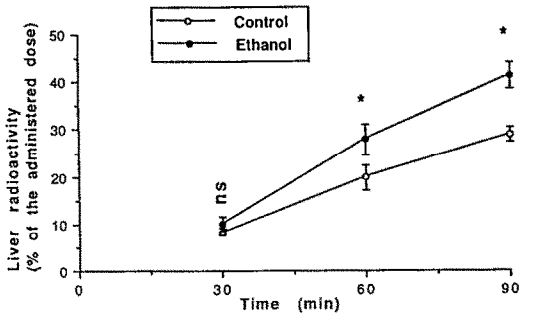

Fig. 2. Effect of an acute ethanol load on the uptake of ^{59}Fe by rat liver as a function of time. Overnight fasted rats received ethanol (50 mmol/kg, i.p.) 10 min before the label. At zero time [^{59}Fe]transferrin was administered intravenously. Each value represents the mean from 5–9 animals, the bars are standard errors of the means. *Indicates significantly different from control group at $P < 0.01$.

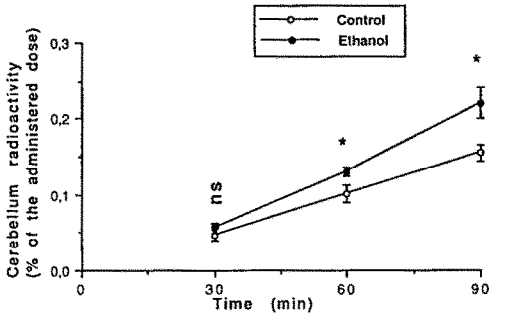

Fig. 3. Effects of an acute ethanol load on the uptake of ^{59}Fe by rat cerebellum as a function of time. Experimental details are as in Fig. 2.

declined rapidly in control rats after the intravenous injection of [^{59}Fe]transferrin, the decline being of a similar order of magnitude as found by others [15]. Compared to control rats, the mean plasma ^{59}Fe activity was significantly reduced ($P < 0.01$) in ethanol-treated animals (by 15 and 22% at 60 and 90 min, respectively).

^{59}Fe was taken up by the liver in a linear fashion from 30 to 90 min after the injection and amounted at 90 min to 28.8% of the injected dose in control rats (Fig. 2). This value is of a similar order of magnitude as previously reported with human transferrin [16]. In ethanol-treated rats, hepatic ^{59}Fe activity was significantly increased (by 40.7 and 43% at 60 and 90 min after the injection of labeled transferrin, respectively) (Fig. 2). The ANOVA test showed significant differences between the slopes of regression lines relating iron uptake and time in these two groups [$F(1.36) = 20.3$; $P < 0.01$].

^{59}Fe uptake by the cerebellum increased in a linear fashion from 30 to 90 min after the injection and at 90 min amounted to 0.15% of the injected dose in control rats (Fig. 3). In ethanol-treated rats, the cerebellar ^{59}Fe activity was significantly increased after the injection of labeled transferrin (by 29 and 42% at 60 and 90 min, respectively)(Fig. 3). Analysis of plasma and cerebellar ^{59}Fe activity allowed the determination of the blood to cerebellar barrier influx rate constant (K_{in}, mL/g-min) for ^{59}Fe. K_{in} was found to be significantly increased in the ethanol-treated rats [38×10^{-4} vs 21×10^{-4}; $F(1,36) = 22.25$, $P < 0.01$].

Effect of allopurinol on non-heme iron levels and iron uptake. As shown in Fig. 4, pretreatment with allopurinol prevented the decrease in the plasma iron level as well as the increase in the hepatic and cerebellar non-heme iron content 100 min after the ethanol load.

Whereas allopurinol alone had no significant effects on the uptake of ^{59}Fe by the liver and cerebellum, pretreatment with allopurinol prevented both the decrease in ^{59}Fe activity in the plasma and the increase in ^{59}Fe uptake by the cerebellum after the ethanol load. At the same time pretreatment with allopurinol significantly reduced the enhancement in hepatic ^{59}Fe radioactivity induced by the ethanol load (Fig. 5).

DISCUSSION

Iron uptake by the liver

The present results show that changes in iron distribution induced by an acute ethanol load are associated with an increase in the hepatic uptake of transferrin-bound iron. Among the multiple mechanisms by which hepatocytes may acquire iron from transferrin, a model in which liver endothelium mediates the hepatic uptake of transferrin has been described [17–19]. In this model a modification of the transferrin structure by the endothelial cells is a prerequisite for the uptake of iron from transferrin by the hepatocyte. In the course of the transendothelial transport, transferrin is partially desialylated allowing it subsequently to be taken up by hepatocytes either via transferrin receptors or via asialoglycoprotein receptors. It has been reported that ethanol *in vitro* promotes the desialylation of transferrin by rat liver endothelium [20], an effect which appears related to the alteration of this endothelium by ethanol. Moreover, Mihas and Tavassoli [21] have recently shown that an *in vivo* acute ethanol administration to rats enhances the desialylation of Tf by rat liver endothelial cells. Such an enhancement in transferrin desialylation could, at least partly, account for the ethanol-induced increase in hepatic iron uptake.

Another model which could account for hepatic iron uptake from transferrin is represented by the reductive release of iron from transferrin at the plasma membrane level [22, 23] involving a plasma membrane redox system termed NADH: ferricyanide oxidoreductase. In hepatocytes, inhibitors of the NADH: ferricyanide oxidoreductase inhibit at the same time iron uptake from transferrin [22]. Furthermore, a low oxygen concentration, which stimulates hepatocyte iron uptake, increases the NADH concentration and the activity of the redox system [22, 24]. In view of this model, ethanol could increase hepatic iron uptake through the reduction of transferrin-associated iron by NADH produced

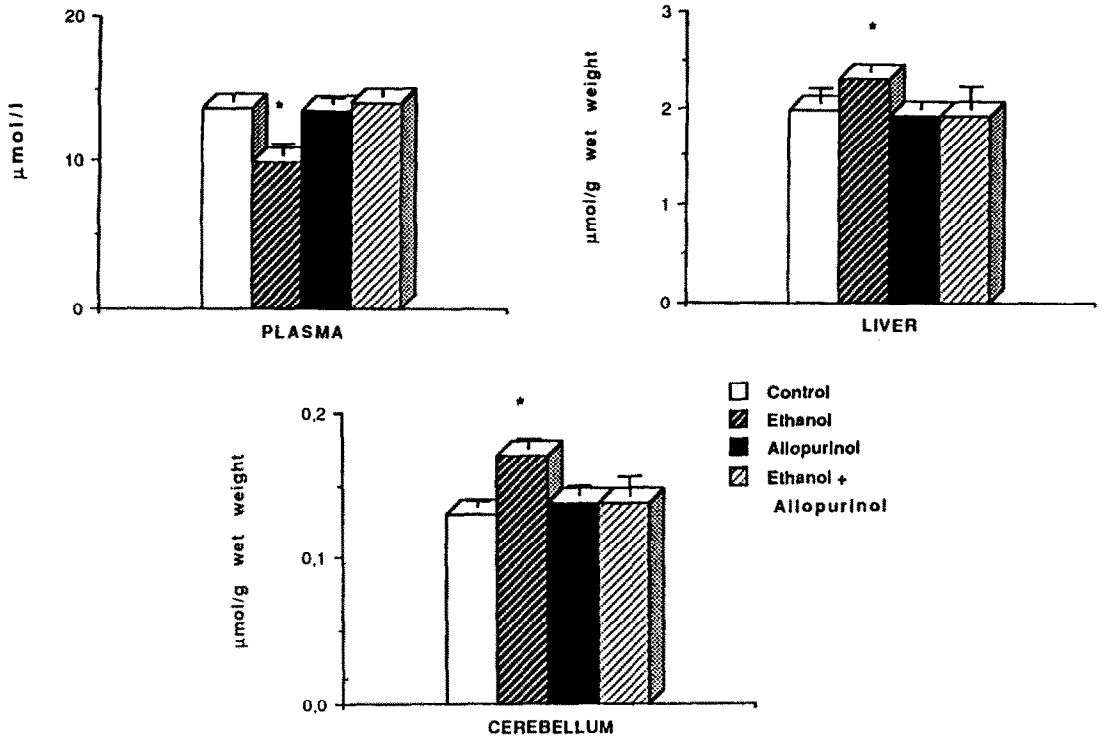

Fig. 4. Effects of ethanol and/or allopurinol on the tissue non-heme iron content. Allopurinol (146 µmol/kg, i.p.) was injected 16 hr and 20 min prior to the ethanol treatment. Ethanol (50 mmol/kg, i.p.) was injected 100 min before sacrifice.* $P < 0.01$ vs controls.

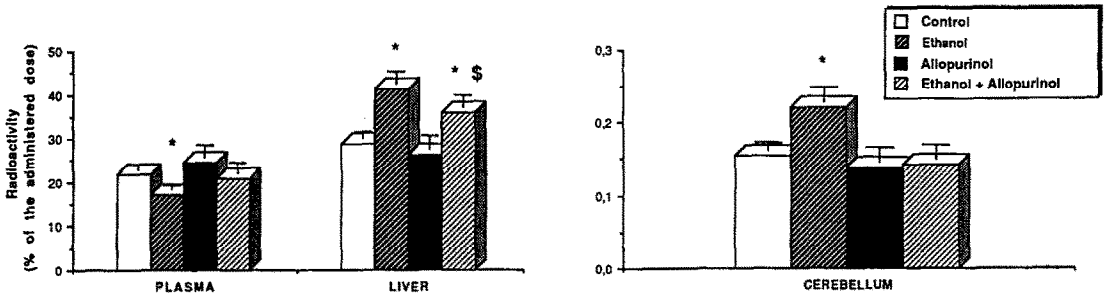

Fig. 5. Effects of ethanol and/or allopurinol on plasma ^{59}Fe disappearance and on liver and cerebellar ^{59}Fe uptake. Allopurinol (146 µmol/kg, i.p.) was injected 16 hr and 20 min prior to ethanol. Ethanol (50 mmol/kg, i.p.) was injected 100 min before sacrifice. [^{59}Fe]Transferrin was administered (i.v.) 10 min after ethanol. Each value is the mean ± SEM from 5–9 animals. *$P < 0.01$ vs controls; §$0.01 < P < 0.02$ versus ethanol-treated animals

during ethanol oxidation by the alcohol dehydrogenase pathway. Moreover it has been reported [25] that ethanol induces hepatic vasoconstriction, leading to hepatic microcirculatory disturbances with resulting focal hypoxia and that the pO_2 is extremely low in some parts of the hepatic tissue after an acute ethanol load [26]. Therefore alterations in pO_2 and in the NADH levels may account for the increase in hepatic iron uptake through the NADH: ferricyanide oxidoreductase mechanism.

We have shown previously that pretreatment with allopurinol provides protection against ethanol-induced hepatic oxidative stress. As a matter of fact allopurinol prevented altogether the ethanol-induced enhancement in liver lipid peroxidation and in non-heme iron as well as the decrease in α-tocopherol, zinc, copper and selenium [9, 10]. This preventive effect of allopurinol could be linked to its inhibitory effect on xanthine oxidase, an oxygen-dependent superoxide-producing enzyme. Using the same

experimental conditions, we presently report that pretreatment with allopurinol reduces the ethanol-induced enhancement in hepatic ^{59}Fe uptake. It can therefore be postulated that a mechanism by which transferrin is induced to release iron could involve free radicals generated in an acidic environment. Support for this hypothesis has come from studies of Brieland and co-workers [27, 28] who showed that PMN-derived O_2^- facilitated the release of Fe^{2+} from transferrin at physiological levels of iron saturation at mildly acidic pH. In our experimental conditions ethanol elicits a hepatic oxidative stress [29]. Furthermore a mild acidosis possibly linked to increased lactic [30] or acetic [31] acid level can be observed following ethanol ingestion. Such alterations may also enhance hepatic iron uptake and thus disturb liver iron homeostastis. The partial prevention of these disturbances by pretreatment with allopurinol suggests that free radical mechanisms contribute to the increase in hepatic iron uptake induced by ethanol. However, whether this contribution is represented by an increased release of transferrin-derived iron or by other, as yet unknown mechanisms, including increased desialylation of transferrin, still remains to be ascertained.

The ethanol induced increase in the hepatic uptake of transferrin-bound iron appears by itself insufficient to explain the observed hepatic accumulation of non-heme iron. Other mechanisms, such as increased iron accumulation resulting from the stimulation of Kupffer cells phagocytic activity [32] are therefore likely to contribute to the ethanol-induced enhancement in the non-heme liver iron content, observed during the present short-term studies. In fact it has been suggested that Kupffer cells release a large part of iron acquired by erythrophagocytosis in the form of ferritin and that ferritin may serve as an intrahepatic carrier of iron between Kupffer cells and hepatocytes [33]. Nevertheless an increment in the iron uptake from transferrin may contribute to the moderate and progressive hepatic iron accumulation frequently observed in human alcoholics. Such an increment has been reported by Chapman *et al.* [34] in human alcoholics with liver diseases as well as in patients with primary biliary cirrhosis. Our data suggest that it could result from a primary effect of ethanol, not directly linked to liver damage.

Iron uptake by the cerebellum

The blood to cerebellar barrier influx rate constant (K_{in}) determined in this study in control Sprague–Dawley rats is significantly higher than previous results concerning iron uptake by the brain of adult Wistar rats [35]. As rat brain capillary transferrin receptors show similar affinities for transferrin from different species [36] our results cannot be related to the use of human transferrin. However one can suggest that endocytosis of transferrin mediated by transferrin receptors display brain region specificity and/or strain specificity. The mechanism of entry of transferrin-bound iron into the brain is mediated via a specific cell surface receptor and involves the transferrin receptors located at the surface of the endothelial cells present at the blood–brain barrier level. Iron enters first in these endothelial cells by means of receptor-mediated endocytosis. Secondly iron is delivered into the interstitial fluid where it is bound by brain-delivered transferrin. Finally it is taken up by receptor-mediated endocytosis into neuronal or glial cells expressing transferrin receptors [37, 38].

A mechanism involving reduction of transferrin-bound Fe^{3+} to Fe^{2+} at the cell surface and subsequent transfer of iron into the cell interior similar to that demonstrated in intact hepatocytes has not yet been demonstrated in brain cells. Such a reduction could however be of non-enzymatic nature and involve physiological cell components such as ascorbate [39] or free radicals acting on acidic destabilizated transferrin [30].

Since our present results show that allopurinol prevents the cerebellar ethanol-induced increase in ^{59}Fe uptake, it can be postulated that oxygen radicals derived from xanthine oxidase activity can be involved in the reduction of transferrin-bound Fe^{3+} to Fe^{2+} at the cell surface. This reduction could be facilitated by the mild acidosis resulting from acute ethanol administration. A 5–7-fold increase in circulatory acetate has been reported 30 min after an acute i.p. administration of ethanol at doses of 0.5 g/kg and higher [40]. Acetate generated from the hepatic oxidation of ethanol is released into the systemic circulation, readily crosses the blood–brain barrier and is actively metabolized in the brain. It has been assumed that organic acids such as lactic and acetic acids cause intracellular acidification due to a rapid influx of the permeable protonated form of organic acids into the cells [41, 42]. Furthermore acute ethanol reduces cerebellar blood flow [43], favouring cellular acidosis and the conversion of xanthine dehydrogenase into the free radical producing oxidase form.

Our results show that an acute ethanol load increases iron uptake by rat liver and cerebellum and suggest the involvement of free radicals in the increased uptake of transferrin-bound iron. Such an increase in iron uptake may contribute to hepatic and cerebellar oxidative damage.

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