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Role of Free Radicals in Primary Nonfunction of Marginal Fatty Grafts from Rats Treated Acutely with Ethanol

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SUMMARY

Acute treatment with one large dose of ethanol, which mimics binge drinking, causes marginal fatty liver and decreases survival significantly after liver transplantation in rats, yet mechanisms remain unclear. Therefore, we evaluated the possible role of free radicals in primary nonfunction caused by acute ethanol. Female donor rats were administered ethanol (5 g/kg orally) 20 hr before explantation, and grafts were stored in UW cold storage solution for 24–42 hr before implantation. Free radicals were trapped with α-(4-pyridyl 1-oxide)-*N*-*tert*-butylnitrone after transplantation, and adducts were detected using electron spin resonance spectrometry. Ethanol increased a carbon-centered radical adduct in bile ~2-fold and elevated serum lipid hydroperoxides ~4-fold. Ethanol also increased transaminase release 3.7-fold and decreased bile production by 55%. Catechin, a free radical scavenger, minimized the increase in free radicals, blunted transaminase release, and elevated bile production significantly, indicating that free radical production plays an important role in ethanol-induced fatty graft injury. $GdCl_3$ (20 mg/kg intravenously), a selective Kupffer cell toxicant, largely blocked the increases in free radical and lipid hydroperoxide production caused by ethanol. In addition, ethanol nearly doubled white blood cell adhesion after transplantation, leading to increased superoxide production in fatty grafts. $GdCl_3$ largely blocked leukocyte adhesion as well as superoxide production. Allopurinol, an inhibitor of xanthine oxidase, also diminished free radical production, blunted transaminase release, and improved bile production in fatty grafts significantly. Taken together, we conclude that free radical formation increases in ethanol-induced fatty grafts due mainly to activation of Kupffer cells and increased adhesion of white blood cells. Antioxidants can effectively block free radical formation and minimize injury to marginal fatty grafts caused by binge drinking.

Orthotopic liver transplantation is recognized as the treatment of choice for acute and chronic end-stage liver disease in adult and pediatric patients; however, a critical shortage of donor organs remains an important problem. Thousands of patients die annually while on waiting lists due to the scarcity of donor organs. One critical problem in liver transplantation is that fatty livers resulting largely from alcohol consumption often fail when used as donor organs (1). For this reason, transplant surgeons sometimes discard fatty livers despite a severe organ shortage. Chronic alcohol consumption causes inflammation, degeneration, necrosis, and cirrhosis in the liver, whereas hydropic degeneration and cell necrosis are also related to primary graft nonfunction (1). Unfortunately, the source of organ donors is mainly accident victims, in whom alcohol consumption is heavily involved (2), and grafts from victims with elevated blood alcohol levels have high rates of primary graft failure (3). Therefore, the mechanisms by which alcohol induces primary graft failure must be understood if the donor pool of usable organs is to be expanded.

Previous studies have shown that an oxygen-dependent reperfusion injury is involved in primary graft nonfunction, and free radicals could be detected within 5 min of completion of implantation surgery (4). Moreover, numerous studies have demonstrated that alcohol consumption increases lipid peroxidation in the liver; pentane, malondialdehyde, and diene conjugates, products of lipid peroxidation, were increased in the liver after both acute and chronic ethanol administration (5, 6), and α-hydroxyethyl radicals derived from alcohol have been detected both *in vitro* (7) and *in vivo*

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ABBREVIATIONS: UW, University of Wisconsin; 4-POBN, α-(4-pyridyl 1-oxide)-*N*-*tert*-butylnitrone; AST, aspartate aminotransferase; MCDP, 10-*N*-methylcarbamoyl-3,7-dimethylamino-10-*H*-phenothiazine; HBSS, Hanks' balanced salt solution; SOD, superoxide dismutase; ANOVA, analysis of variance; PMA, phorbol-12-myristate-13-acetate; MDA, mitochondrial malondialdehyde.

(8). Thus, it is likely that alcohol increases oxidative stress after transplantation.

Organ donation is often associated with accidents that frequently involve binge drinking. Acute ethanol treatment mimics binge drinking and provides an inexpensive and convenient model. It is known that even a single large dose of ethanol significantly increases hepatic lipid content (9), and previous work from this laboratory has shown that one single large dose of ethanol decreased survival after transplantation from ~90 to 30% (10). Accordingly, the effects of ethanol exposure on free radical formation and graft injury after liver transplantation were studied after one large, acute dose of ethanol to mimic binge drinking.

Materials and Methods

Animals and liver transplantation. Inbred female Lewis rats (190–230 g) were used in liver transplantation experiments to prevent immunological interference. Donor rats were treated with ethanol (5 g/kg body weight orally) 20 hr before explantation. $GdCl_3$ (20 mg/kg) dissolved in acidic saline, pH 3.0–3.5, was administered to some donors intravenously 24 hr before surgery to destroy Kupffer cells (11). In some experiments, donor rats were administered allopurinol (100 mg/kg orally), a xanthine oxidase inhibitor, 24 and 1 hr before explantation (12). Rats were anesthetized with Metofane, and liver transplantation was performed using the technique described by Zimmermann and Kamada (13). Briefly, heparin (200 IU) in 0.5 ml of Ringer's solution was injected into the subhepatic vena cava, a 4-mm-long stent prepared from polyethylene tubing (PE-50, Clay Adams, Parsippany, NJ) was inserted into the common bile duct and secured with a 6–0 suture, and livers were flushed *in situ* with 5 ml of Ringer's solution (0–4°) followed by 3 ml of UW cold storage solution (100 mM KH_2PO_4, 5 mM $MgSO_4$ 100 mM potassium lactobionate, 1 mM allopurinol, 3 mM glutathione, 5 mM adenosine, 30 mM raffinose, 50 g/liter of hydroxyethyl starch, 40 units/liter of insulin, 16 mg/liter of dexamethasone, 200,000 units/liter of penicillin, pH 7.4; 320–330 mOsM). Venous cuffs prepared from 14-gauge intravenous catheters were placed in the subhepatic vena cava and the portal vein, and grafts were stored in UW solution at 0–4° for the times indicated in the figure legends. For implantation, the liver of the recipient was removed after clamping the suprahepatic vena cava, portal vein, and subhepatic vena cava, and grafts were implanted by connecting the suprahepatic vena cava with a running suture and then inserting cuffs into the appropriate vessels and securing them with 6–0 silk suture. The bile duct was anastomosed with an intraluminal stent. Implantation surgery required <50 min; during this time, the portal vein was clamped for 16–22 min. Survival was assumed to be permanent when rats were alive at 30 days after surgery.

Detection of free radical adducts. To assess free radical formation by liver grafts, the spin trapping reagent 4-POBN (1 g/kg body weight) was dissolved in 0.5 ml of normal saline and injected slowly into the tail vein after opening the vascular clamps. Bile was collected for 1 hr after implantation, via a cannula (PE-50) placed in the common bile duct, into 50 μl of 30 mM dipyridyl on ice to prevent *ex vivo* free radical formation, and samples were stored on dry ice until analysis. Bile samples were thawed, placed in a quartz ESR cell, and scanned repeatedly until the interfering ascorbate semiquinone signal disappeared (~1 hr). Free radical adducts were detected with a Bruker ESP 106 ESR spectrometer (Bruker Instrument, Billerica, MA). Instrument conditions were 20-mW microwave power; 1.0-G modulation amplitude, and 80-G scan range. Spectral data were stored on an IBM-compatible computer and were analyzed for ESR hyperfine coupling constants by computer simulation (8). The magnitude of the six-line signal was measured at identical gains and expressed in arbitrary units (1 unit = 1 cm of chart paper).

Measurement of lipid hydroperoxides and serum AST. Serum lipid hydroperoxides were measured on the basis of the formation of methylene blue from the chromogen MCDP by a redox reaction of hydroperoxides with hemoglobin as a catalyst (14). Authentic lipid hydroperoxides were used to generate standard curves.

To measure AST release due to liver injury, blood samples were collected postoperatively from the inferior vena cava for the first 3 hr after implantation. Serum was obtained by centrifugation and stored at −20°, and AST activity was determined by standard enzymatic methods (15).

Bile production. The common bile duct was cannulated with polyethylene tubing (PE-50) in some experiments, and bile was collected for 3 hr after organ implantation. Rates of bile production were calculated from the length of time and the volume of bile and normalized using the DNA content of the liver tissue to rule out the possible influence of ethanol-induced fat droplets on liver weight. DNA content of the liver tissue was measured using the bisbenzimidazole method as described previously (16).

Mitochondrial respiration and malondialdehyde and lipid hydroperoxide production. Mitochondria were isolated from livers by standard procedures of differential centrifugation (17). Isolated mitochondria were incubated in buffer containing 100 mM KCl, 50 mM sucrose, 20 mM Tris·HCl, 5 mM Tris-phosphate, and 10 μM rotenone. Oxygen uptake was measured in a closed vessel (2.0 ml) with a Clark-type oxygen electrode after the addition of succinate (2.5 mM) and ADP (0.25 mM). In some experiments, mitochondria were sonicated, and malondialdehyde was measured in trichloroacetic acid extracts using the thiobarbituric acid method (18). Lipid hydroperoxides in sonicated mitochondrial suspensions were measured on the basis of absorbance of methylene blue formed from MCDP as described above, and mitochondrial protein was determined according to the method of Lowry (19).

Isolation of adherent white blood cells. Thirty minutes after implantation, some liver grafts were perfused with 250 ml of HBSS containing calcium and magnesium to remove blood as described in detail previously (20). Briefly, grafts were perfused with 400 ml of HBSS devoid of calcium and magnesium but containing 1 mM EGTA and 1 unit/ml heparin (calcium-free buffer) at 37°. Previous work demonstrated that essentially all adherent white blood cells could be isolated from the liver with this procedure (20). Effluent (400 ml) was collected and centrifuged at 500 × *g* for 10 min to pellet previously adherent cells, and contaminating red blood cells were lysed with hypotonic NH_4Cl. White blood cells were counted on a hemocytometer, and cytospin preparations were stained with Wright-Giemsa to obtain differential white blood cell counts.

Measurement of superoxide. Production of superoxide by white blood cells isolated from grafts was determined from the SOD inhibitable reduction of ferricytochrome *c* (21). Briefly, a suspension of ~3×10^6 white blood cells was incubated in HBSS buffer at 37° with 80 μM cytochrome *c* in the presence and absence of 3.2×10^{-9} M PMA, which stimulates NADPH oxidase via protein kinase C. SOD was added to one tube (SOD+) and an equal volume of buffer was placed in another tube (SOD−). To terminate the reaction, SOD was added to the SOD− tube at the end of incubation. White blood cells were pelleted by centrifugation (300 × *g*) at 0–4° for 7 min, and the amount of reduced cytochrome c in the supernatant was measured spectrophotometrically at 550 nm.

Statistical analysis. All groups were compared using Student's *t* test or ANOVA plus Student-Newman-Keuls test as appropriate, and differences were considered significant at the $p < 0.05$ level.

Results

Ethanol increases free radical formation and graft injury after liver transplantation. Graft injury was assessed by release of AST into the blood after liver transplantation. Serum AST levels were ~100 units/liter in both the

control and acute alcohol groups before restoration of blood flow; however, values increased gradually to ~600 units/liter over 3 hr after implantation of grafts stored in UW solution for 24 hr (Fig. 1A). AST release by grafts from rats treated acutely with ethanol (5 g/kg body weight orally) was nearly 4-fold greater than that of untreated controls, reaching values ~2200 units/liter. Bile production, an indicator of graft function, increased gradually after implantation and reached a steady state value of ~6 μl/mg hepatic DNA/hr at 2 hr after restoration of blood flow. Bile production was decreased significantly by >50% in grafts from ethanol-treated rats (Fig. 1B).

A six-line ESR spectrum due to a 4-POBN/radical adduct was detected in bile samples from all livers at 1 hr after transplantation. Acute ethanol treatment of the donor rat before liver explantation increased the signal amplitude by ~2-fold (Fig. 2). Computer simulation of the spectrum was accomplished using hyperfine coupling constants of a^N = 15.70 G and a^H = 2.66 G for a single radical species. Such coupling constants are characteristic of a carbon-centered 4-POBN radical adduct and match closely values (a^N = 15.63 G and a^H = 2.73 G) obtained from bile of rats administered spin-trap and oxidized polyunsaturated fatty acids (22).

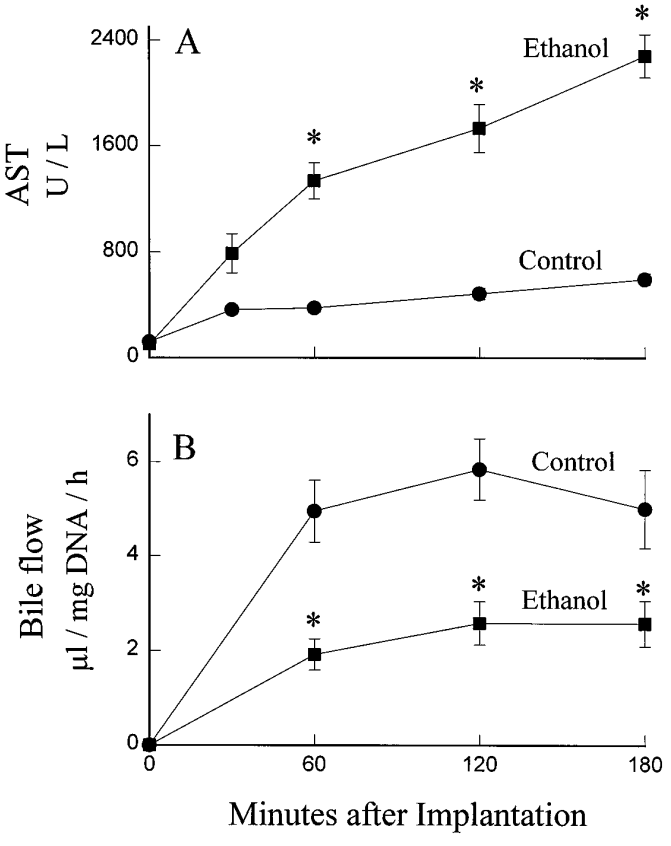

Fig. 1. Effects of acute ethanol treatment on AST release and bile production after liver transplantation. Donor rats were administered ethanol (5 g/kg orally) 20 hr before explantation, and grafts were stored in UW solution for 24 hr before implantation. Blood samples were collected postoperatively from the inferior vena cava, and bile was collected via a cannula placed in the common bile duct for the first 3 hr after implantation. AST activity was determined by standard enzymatic methods (15). Bile production was normalized using DNA content of the graft. A, AST release. B, Bile production. Values are mean ± standard error (six or seven animals in each group; $p < 0.01$ by ANOVA). *, $p < 0.05$ compared with controls.

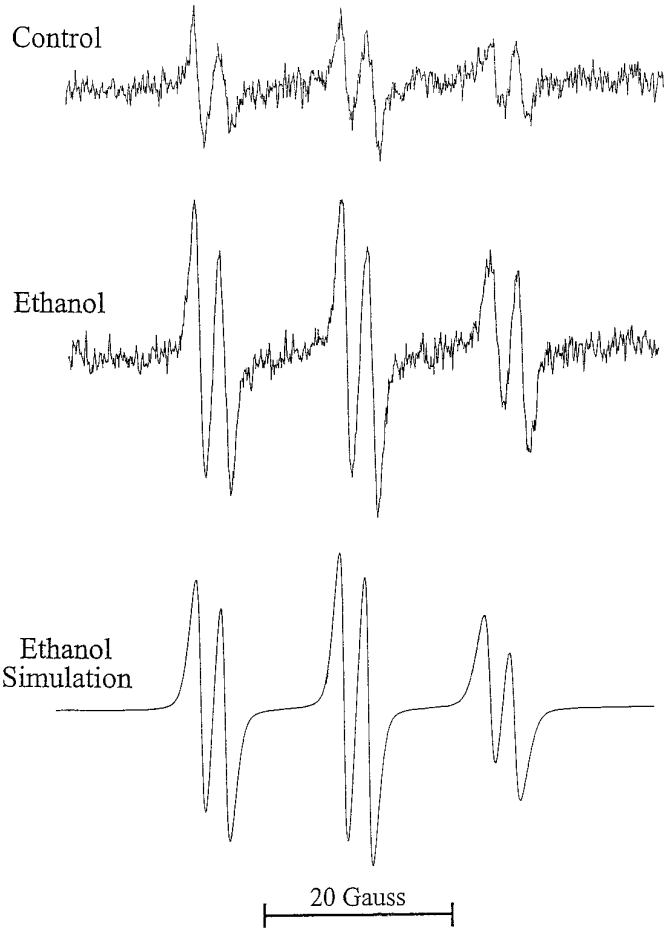

Fig. 2. Effect of acute ethanol treatment on ESR spectra of free radical adducts in bile. Conditions were as represented in legend to Fig. 1 except grafts were stored in UW solution for 42 hr before implantation. The spin-trapping reagent 4-POBN (1 g/kg) was dissolved in 0.5 ml of normal saline and injected slowly into the tail vein of the recipient on opening of the vascular clamps. Bile excreted during the first hour after implantation was collected, via a cannula (PE-50) placed in the common bile duct, into 50 μl of 30 mM dipyridyl on ice to prevent *ex vivo* free radical formation and stored on dry ice until analysis. Free radical adducts were detected using a Bruker ESP 106 ESR spectrometer with the instrument conditions of 20-mW microwave power, 1.0-G modulation amplitude, and 80-G scan range. Typical spectra: *Top*, control. *Middle*, ethanol. *Bottom*, simulation of ESR spectra of radical adducts from the ethanol-treated group.

Compounds with antioxidant properties decrease free radical formation and graft injury caused by ethanol. Because ethanol increases free radical formation and graft failure after transplantation, we investigated whether a free radical scavenger could diminish graft injury after transplantation. Catechin, a flavonoid that scavenges free radicals and singlet oxygen (23), was administered to the recipient on completion of implantation surgery. The graft was also rinsed with lactated Ringer's solution containing catechin. This treatment blunted free radical adduct formation with values reaching only~20% of those observed in grafts from ethanol-treated rats (Fig. 3). When fatty grafts were rinsed with Carolina Rinse solution, which contains antioxidants and several compounds that inhibit the generation of free radicals (glutathione, desferrioxamine, and allopurinol) before implantation, free radical adduct formation was reduced by ~50%. Catechin also reduced postoperative serum AST

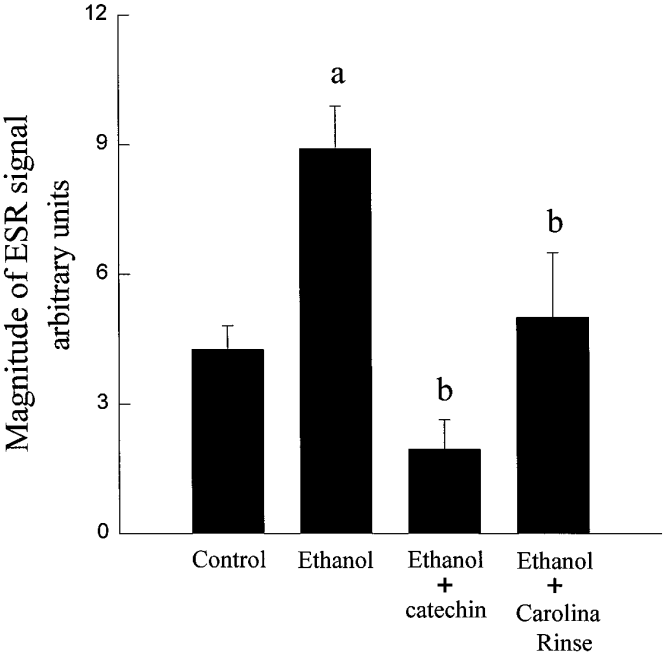

Fig. 3. Effects of acute ethanol and free radical scavengers on free radical formation. Grafts were stored in UW solution for 42 hr before implantation, and free radicals were detected as described in legend to Fig. 2. In some experiments, catechin (30 mg/kg intravenously) was administered to recipients on initiation of implantation surgery, and grafts were rinsed with 5 ml of Ringer's solution containing 400 μM catechin. Some grafts were rinsed with 5 ml of Carolina Rinse solution before implantation. Values are average magnitude of the low-field line (*first line from left*, see Fig. 2) of ESR spectra ± standard error (four or five animals in each group; $p < 0.001$ by ANOVA). *a*, $p < 0.05$ compared with control. *b*, $p < 0.05$ compared with the ethanol group.

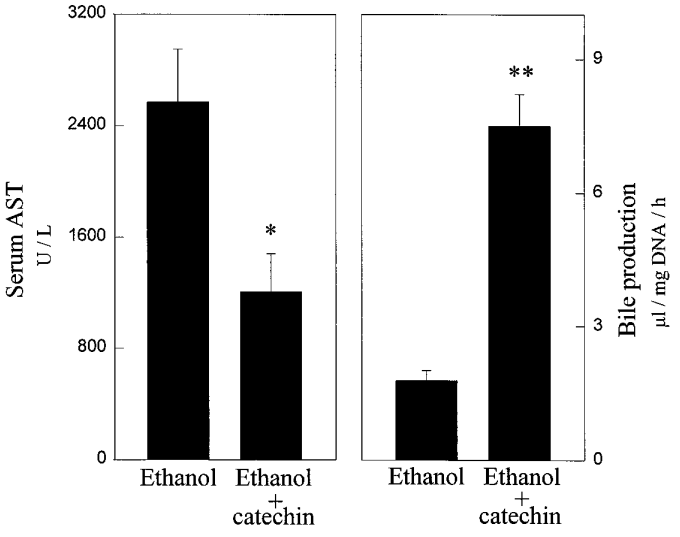

Fig. 4. Effects of catechin on AST release and bile production by fatty grafts. Conditions were as represented in legend to Fig. 3. Catechin (30 mg/kg intravenously) was administered to recipients on initiation of implantation surgery, and grafts were rinsed with 5 ml of Ringer's solution containing 400 μM catechin. Blood samples were collected postoperatively from the inferior vena cava, and bile was collected via a cannula placed in the common bile duct for the first 3 hr after implantation. Serum AST was determined by standard enzymatic methods (15). *Left*, AST release. *Right*, bile production. Values are mean ± standard error (four or five animals in each group). *, $p < 0.05$; and **, $p < 0.01$, compared with control by Student's *t* test.

release by 50% and increased bile production by 4.2-fold in grafts from rats treated with ethanol acutely, reflecting diminished graft injury (Fig. 4.).

Inhibition of xanthine oxidase and destruction of Kupffer cells decrease free radical production in a binge drinking model. Xanthine oxidase, which is activated by ischemia/reperfusion, could be involved in free radical formation on reperfusion. Therefore, we investigated the effect of allopurinol, a potent inhibitor of xanthine oxidase and a free radical scavenger (12). Allopurinol administered to recipients just before implantation and added to the rinse solution did not alter free radical formation by fatty grafts (data not shown), indicating that it did not act as a free radical scavenger *per se* under these conditions. However, it largely blocked the increase in radical formation caused by ethanol when it was administered to both donors and recipients and used to rinse the graft (Fig. 5). Allopurinol also reduced postoperative serum AST release by ~40% ($p < 0.01$) and increased bile production by 1.8-fold ($p < 0.05$) in grafts from rats treated acutely with ethanol, reflecting diminished graft injury (data not shown.)

$GdCl_3$, which selectively destroys Kupffer cells (11), largely blocked free radical production caused by ethanol (Fig. 5). Moreover, lipid hydroperoxides, end products of lipid peroxidation, increased gradually from 4.2 to 7.3 nmol/ml of serum by 1 hr after transplantation. Ethanol increased peak lipid hydroperoxide release by ~4-fold, an effect that was also largely blocked by $GdCl_3$ (Fig. 6).

Destruction of Kupffer cells diminishes white blood cell adhesion and superoxide production caused by ethanol. Adherent neutrophils and monocytes in ischemic-reperfused tissue could be an important source of free radicals; therefore, adherent white blood cells were isolated from grafts 30 min after transplantation (20). Approximately 7.1×10^5 adherent white blood cells/mg hepatic DNA were isolated from grafts from untreated rats, a value increased 1.9-fold by acute ethanol treatment. The removal of Kupffer cells with $GdCl_3$ blunted this effect by ~60% (Fig. 7, *top*). Lymphocytes constituted 76% of white blood cells isolated from control grafts after transplantation, a value decreased to 68% ($p < 0.05$) by ethanol treatment. In contrast, monocytes and neutrophils, cells responsible for superoxide production, constitute ~14 and ~10% of white blood cells, respectively. Ethanol increased monocytes slightly to 20% ($p < 0.05$) but did not alter neutrophils or other white blood cells.

Superoxide production in cells from grafts of control rats was 0.9 nmol/10^6 cells/30 min in the presence of PMA (Fig. 7, *middle*), which is close to values reported elsewhere (24). Interestingly, superoxide production in cells from control rats was not significantly different in the presence or absence of PMA, indicating that adherent cells are activated by transplantation (Fig. 7, *middle*). Ethanol treatment did not significantly alter rates of superoxide production per cell in the presence or absence of PMA (Fig. 7, *middle*). However, in the absence of PMA, superoxide production per white blood cell from $GdCl_3$-treated rats was significantly lower than values from the control or ethanol-treated group, indicating that Kupffer cells are involved in activation of neutrophils and monocytes after liver transplantation.

Although ethanol did not significantly alter superoxide production per unit of leukocyte isolated (Fig. 7, *middle*), it

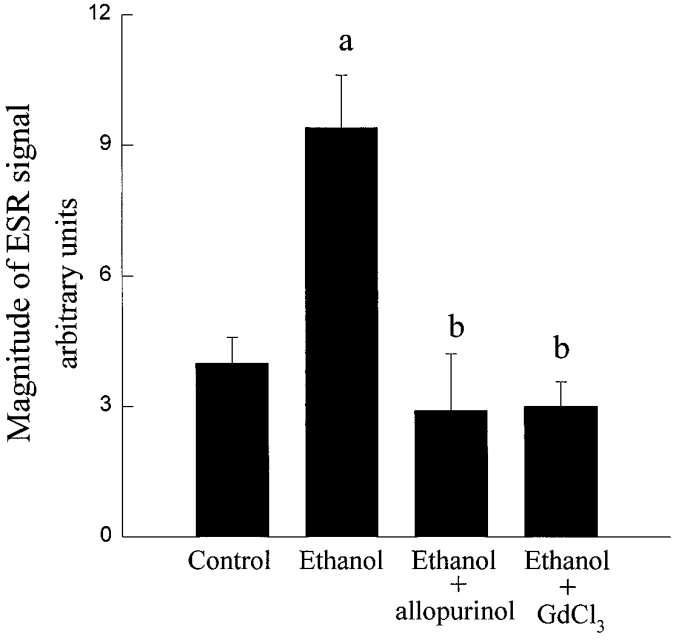

Fig. 5. Effects of allopurinol and $GdCl_3$ on free radical formation after transplantation of fatty grafts due to acute ethanol. Conditions were as represented in legend to Fig. 2. Some donors were administered $GdCl_3$ (20 mg/kg intravenously) 24 hr before explantation. Allopurinol (100 mg/kg orally) was administered to some donors 24 and 1 hr before explantation while the recipient was treated with the same dose 1 hr before implantation. Grafts were rinsed with 5 ml of Ringer's solution containing 4 mM allopurinol before implantation. Values are mean ± standard error (three or four animals in each group; $p < 0.001$ by ANOVA). *a*, $p < 0.05$ compared with control group. *b*, $p < 0.05$ compared with ethanol group.

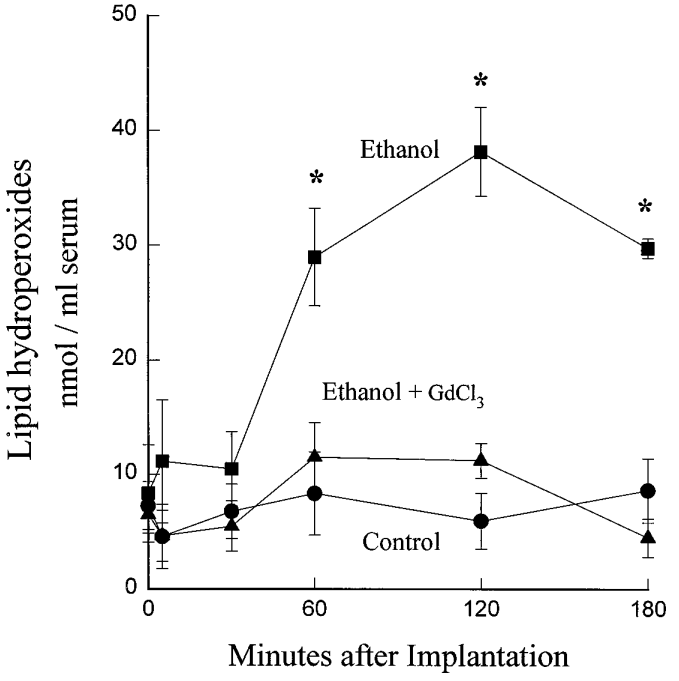

Fig. 6. Effects of acute ethanol and $GdCl_3$ on lipid hydroperoxide production. Conditions were as represented in legend to Fig. 1. Serum hydroperoxides were measured based on the absorbance of methylene blue at 650 nm formed from MCDP as described in the text. Values are mean ± standard error (four animals in each group; $p < 0.01$ by ANOVA). *, $p < 0.05$ compared with control and $GdCl_3$ groups.

increased leukocyte adhesion significantly (Fig. 7, *top*). As a result, superoxide production by adherent white blood cells per unit of liver tissue was increased 2.2–2.3-fold by ethanol (Fig. 7, *bottom*). $GdCl_3$ minimized the increase in superoxide production in the presence of PMA, but the effect was ~2-fold larger in the absence of PMA; therefore, destruction of Kupffer cells decreases free radical production by adherent leukocytes, with reduced adhesion and prevention of activation of leukocytes each contributing approximately equally to the phenomenon (Fig. 7, *bottom*).

MDA and lipid hydroperoxide production were increased by transplantation but not affected by ethanol treatment. State 3 rates of oxygen uptake were ~150 nmol/min/mg of protein in mitochondria isolated from nontransplanted control livers, and respiratory control ratios (state 3/state 4) were 3.8, indicating that mitochondria isolated under these conditions were well coupled. After transplantation, state 3 rates of oxygen uptake were decreased to 106 nmol/min/mg of protein, and respiratory control ratios were reduced to 2.3 in mitochondria isolated from livers not receiving ethanol ($p < 0.05$ compared with nontransplanted livers). However, mitochondrial oxygen uptake and respiratory control ratios after transplantation were not statistically different between ethanol-treated and -untreated grafts. Therefore, inhibition of mitochondrial respiration was primarily due to transplantation and was unaffected by ethanol.

MDA production in mitochondria isolated from untreated livers was 3.3 nmol/mg of protein (Table 1) before transplantation. One hour after transplantation, MDA production was increased in both the control and ethanol-treated groups ~2-fold by transplantation ($p < 0.05$ compared with the nontransplanted group, Table 1); however, MDA production after transplantation was not statistically different between ethanol-treated and -untreated grafts ($p > 0.05$). A similar tendency was observed in lipid hydroperoxide production. These results support the hypothesis that mitochondria do not play an important role in ethanol-induced free radical production after liver transplantation.

Discussion

Acute ethanol treatment increases free radical formation after liver transplantation. Previous studies showed that free radical formation is involved in alcoholic liver disease; products of lipid peroxidation, such as pentane, malondialdehyde, and diene conjugates, were increased in the liver after ethanol administration (5, 6), and α-hydroxyethyl radicals derived from ethanol have been detected both *in vivo* and with microsomes (7, 8). Moreover, free radicals were detected 5 min after transplantation of grafts from untreated rats (4). Therefore, it is possible that ethanol and transplantation increase free radical formation additively or synergistically, thus exacerbating graft injury. Consistent with this idea, chronic ethanol treatment increased free radical formation after ischemia/reoxygenation in the perfused liver (25), and SOD/catalase-insensitive free radicals, most likely from lipid hydroperoxides, were detected in fatty grafts from rats treated chronically with ethanol (26). Because organ donation is often associated with accidents and suicides, which frequently involve binge drinking, the effects of ethanol exposure on free radical formation and graft injury after

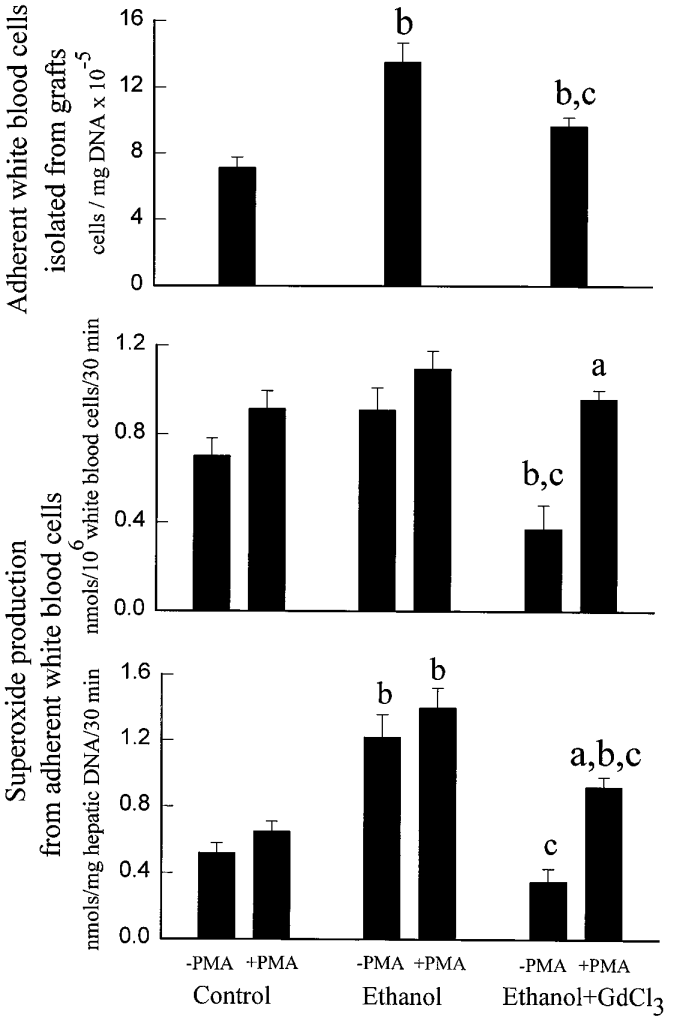

Fig. 7. Effects of acute ethanol and $GdCl_3$ treatment on superoxide production. Conditions were as represented in legend to Fig. 1. Thirty minutes after implantation, adherent white blood cells were isolated from grafts as described in the text. Cells were preincubated in the presence or absence of PMA (*+PMA* and *−PMA*, respectively), and production of superoxide was determined from the superoxide dismutase inhibitable reduction of ferricytochrome *c* (21). Rates of superoxide production in white blood cells isolated from whole blood of untransplanted normal rats were 0.20 in the absence of PMA and 1.35 nmol/10^6 cells in the presence PMA. *Top*, leukocytes isolated per unit of liver. *Middle*, superoxide production per unit of isolated leukocytes. *Bottom*, superoxide production per unit liver. Values are mean ± standard error (four or five in each group; $p < 0.001$ by ANOVA). *a*, $p < 0.05$ compared with −PMA group. *b*, $p < 0.05$ compared with the appropriate control. *c*, $p < 0.05$ compared with the appropriate ethanol group.

liver transplantation were studied here after one large, acute dose of ethanol. Free radical adduct formation was doubled by acute ethanol treatment in female rats (Fig. 2). Based on coupling constants, we concluded that radical adducts detected here are lipid radicals. Consistent with direct free radical measurement, lipid hydroperoxides, the products of lipid hydroperoxyl radicals that are SOD/catalase insensitive, were increased significantly in grafts from rats treated acutely with ethanol (Fig. 6). These results are consistent with the hypothesis that acute ethanol treatment increases free radical formation, most likely by increasing lipid radical formation, in an animal model that mimics binge drinking.

An important finding of this study is that catechin, a free radical and singlet oxygen scavenger (23), blocked ethanol-stimulated free radical formation (Fig. 3) and minimized reperfusion injury in grafts from ethanol-treated rats (Fig. 4). We concluded that free radical production is causally related to ethanol-induced graft injury, and compounds with radical trapping properties could be used to minimize injury and improve transplantation outcomes.

How does ethanol increase free radical formation after liver transplantation? Three sources of free radical formation in grafts from ethanol-treated rats have been considered in this study, including Kupffer cells, white blood cells, and xanthine oxidase. Kupffer cells, the resident macrophages in the liver, are activated by ischemia/reoxygenation (27), and superoxide production by Kupffer cells isolated from livers after *in vivo* ischemia/reperfusion was increased nearly 6-fold (24). Kupffer cells constitute ~80% of fixed macrophage in the body (28) and, therefore, represent an important source of free radicals after ischemia/reoxygenation. Alcohol could activate Kupffer cells, thus increasing superoxide production on reperfusion (Fig. 8). However, the effect of ethanol treatment on Kupffer cells is controversial. Acute ethanol treatment increased superoxide production by Kupffer cells 3.5-fold (29) and stimulated carbon uptake in perfused livers (30). Paradoxically, ethanol treatment decreased endotoxin-stimulated superoxide production in the perfused liver (31). This paradox is probably due to the temporal effects of ethanol on Kupffer cells. A recent study showed that endotoxin-induced increases in intracellular calcium in Kupffer cells were blunted at 2 hr but elevated 2.5-fold at 24 hr after ethanol treatment (32). In the current study, destruction of Kupffer cells largely blocked ethanol-induced free radical production after transplantation (Figs. 5 and 6), indicating that ethanol increases free radical production, most likely by activating Kupffer cells under these conditions. The mechanisms by which acute ethanol treatment stimulates Kupffer cells, however, remain unclear. One possibility is that ethanol increases blood endotoxin (33), which primes or activates Kupffer cells in donors. Indeed, pretreatment with antibiotics largely blunted increases of intracellular calcium in Kupffer cells isolated from rats treated acutely with ethanol, supporting the hypothesis that ethanol affects Kupffer cells by increasing endotoxin in the blood (32). Moreover, ethanol causes hypoxia in the liver (34), and subsequent reoxygenation is known to activate Kupffer cells (27) (Fig. 8). Taken together, it is concluded that Kupffer cell activation plays an important role in free radical production in ethanol-induced fatty grafts.

Several pathways could be involved in the elevation of free radical formation by Kupffer cells in ethanol-induced fatty grafts. First, protein kinase C is stimulated by reperfusion, thus activating NADPH oxidase to produce superoxide radicals (35) (Fig. 8). Indeed, SPC-100270, which selectively inhibits protein kinase C, reduced graft injury after transplantation (36). Moreover, elevated synthesis of eicosanoids on Kupffer cell activation could produce free lipid radical intermediates (37). In this study, allopurinol, an inhibitor of xanthine oxidase, significantly reduced free radical formation after transplantation of fatty grafts (Fig. 5), indicating that activation of xanthine oxidase also plays an important role in radical production. A recent study showed that conversion of xanthine dehydrogenase to xanthine oxidase after cold stor-

TABLE 1

Effect of acute ethanol treatment and liver transplantation on mitochondrial respiration, malondialdehyde, and lipid hydroperoxide formation

Mitochondria were isolated from livers either before cold storage or 1 hr after implantation. Some donor rats were pretreated with ethanol (orally; 5 g/kg of body weight) 20 hr before explantation. Mitochondrial oxygen uptake and respiratory control ratios were measured as described in Materials and Methods. Malondialdehyde was detected using the thiobarbituric acid method (18), and lipid hydroperoxides were measured based on the optical density of methylene blue at 650 nm formed from MCDP as described in Materials and Methods. Values are mean ± standard error (four livers in each group). All groups were compared using ANOVA plus Student-Newman-Keuls *post hoc* test. The *p*-values for ANOVA were 0.008 for mitochondrial oxygen uptake, 0.01 for respiratory control ratio, 0.04 for malondialdehyde, and 0.033 for lipid hydroperoxide.

	O_2 uptake state 3	Respiratory control ratio	Malondialdehyde	Lipid hydroperoxide
	nmol/min/mg of protein		*nmol/mg of protein*	
Untransplanted control	152 ± 5	3.8 ± 0.4	3.29 ± 0.30	36.3 ± 7.3
Transplanted control	107 ± 8[a]	2.3 ± 0.3[a]	6.56 ± 0.30[a]	61.8 ± 8.6
Transplanted ethanol	83 ± 20[a]	1.8 ± 0.3[a]	7.00 ± 1.35[a]	73.3 ± 15.0

[a] $p < 0.05$ compared with untransplanted controls.

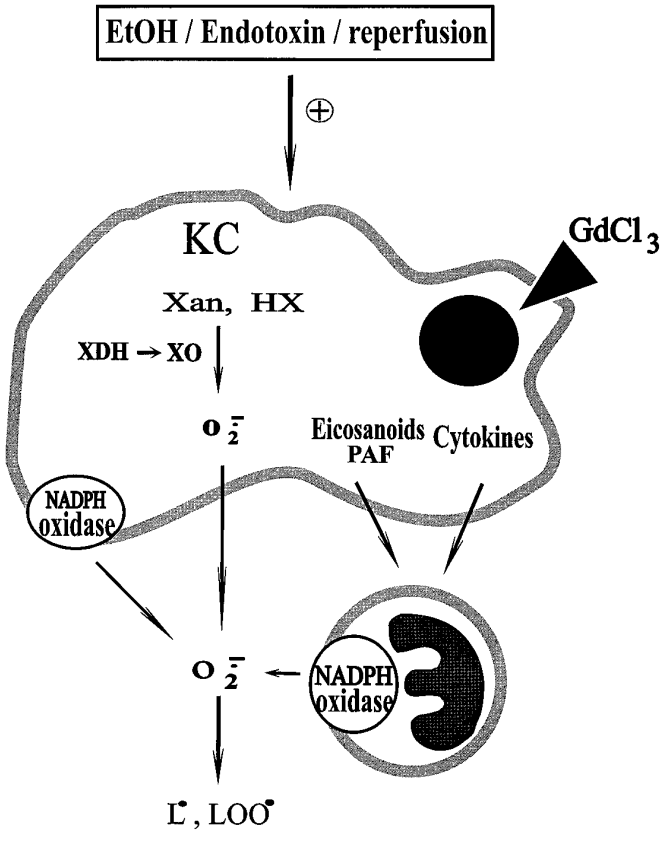

Fig. 8. Diagram depicting the possible mechanisms by which free radicals are formed and grafts injured after transplantation of fatty grafts produced by binge drinking. It is proposed that acute ethanol treatment (*binge drinking*) exacerbates graft injury by the following mechanisms: first, ethanol activates Kupffer cells, thus increasing formation of free radicals via NADPH oxidase and xanthine oxidase. Second, oxidants stimulate Kupffer cells to produce chemotactic and vasoactive mediators such as eicosanoids, platelet activating factor, and cytokines, which disturb the hepatic microcirculation; increase white blood cell adhesion; and activate adherent polymorphonuclear cells to produce free radicals, which further exacerbates lipid peroxidation. $GdCl_3$ destroys Kupffer cells, allopurinol inhibits xanthine oxidase, and catechin scavenges free radicals, thereby minimizing ethanol-induced graft injury. *KC*, Kupfer cell; *EtOH*, ethanol; O_2^-, superoxide radical; $L^\cdot$, lipid radical; $LOO^\cdot$, lipid peroxde radicals; *Xan*, xanthine, *HX*, hypoxanthine; XPH, xanthine dehydrogenase; *XO* xanthine oxidase; *PAF*, platelet-activating factor.

age is very slow in parenchymal cells but much more rapid in Kupffer cells (38); therefore, Kupffer cells may also produce superoxide via xanthine oxidase (Fig. 8).

In addition, chemotactic and vasoactive mediators (e.g., leukotrienes, thromboxanes, and platelet-activating factor) released from Kupffer cells could increase adhesion and activation of white blood cells (Fig. 8). Oxidative stress activates nuclear factor-κB, a transcription factor responsible for gene expression of cytokines, such as tumor necrosis factor-α and interleukin-1, in Kupffer cells (39, 40). These cytokines induce adhesion molecules such as E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 in endothelial cells, leading to increased white blood cell adhesion (41). Indeed, ethanol increases intercellular adhesion molecule-1 expression after liver transplantation (42), and in the current study, it was shown that both white blood cell adhesion and superoxide production after transplantation were doubled in fatty grafts from rats treated acutely with ethanol (Fig. 7). Further, destruction of Kupffer cells not only reduced the adhesion of white blood cells but also prevented their activation (Fig. 7), most likely due to decreased release of mediators required for the priming and activation of neutrophils such as platelet-activating factor, leukotriene-B_4, and tumor necrosis factor-α (Fig. 8). Therefore, destruction of Kupffer cells decreases superoxide production not only directly by the macrophages themselves but also indirectly through reduction in white blood cell adhesion and neutrophil activation.

In conclusion, acute ethanol treatment enhances free radical formation after transplantation by mechanisms involving activation of Kupffer cells, increases in white blood cell adhesion, and elevation of xanthine oxidase. Antioxidants effectively diminish free radical formation, thus reducing graft injury in a model of binge drinking.

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