Peroxisomes Are Involved in the Swift Increase in Alcohol Metabolism[1]

BLAIR U. BRADFORD, NOBUYUKI ENOMOTO, KENICHI IKEJIMA, MICHELLE L. ROSE, HEIDI K. BOJES, DONALD T. FORMAN and RONALD G. THURMAN

Laboratory of Hepatobiology and Toxicology, Department of Pharmacology (B.U.B., N.E., K.I., M.L.R., H.K.B., R.G.T.), and Department of Pathology and Laboratory Medicine (D.T.F.), University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

ABSTRACT

The purpose of this study was to determine whether catalase-dependent alcohol metabolism is activated by alcohol (i.e., swift increase in alcohol metabolism). When ethanol or the selective substrate for catalase, methanol, was given (5.0 g/kg) in vivo 2 to 3 h before liver perfusion, methanol and oxygen metabolism were increased significantly. This increase was blocked when the specific Kupffer cell toxicant $GdCl_3$ was administered 24 h before perfusion. These data support the hypothesis that catalase-dependent alcohol metabolism is activated by acute alcohol and that Kupffer cells are involved. Ethanol treatment in vivo increased ketogenesis from endogenous fatty acids nearly 3-fold and increased plasma triglycerides and hepatic acyl CoA synthetase activity; all increases were blocked by $GdCl_3$. These findings support the hypothesis that ethanol increases H_2O_2 supply for catalase-dependent alcohol metabolism by increasing fatty acid supply. Infusion of oleate stimulated oxygen uptake 1.5-fold and methanol metabolism 4-fold, but these parameters were not altered by $GdCl_3$. Moreover, the effects of ethanol treatment were blocked by the cyclooxygenase inhibitor indomethacin, and prostaglandin E_2 (PGE_2) was increased more than 200% in media from cultured Kupffer cells from rats treated with ethanol in vivo. Furthermore, lipoprotein lipase activity in retroperitoneal fat pads, which is known to be inhibited by PGE_2, was reduced 70% by ethanol. These data are consistent with the hypothesis that Kupffer cells play a key role in activation of catalase-dependent alcohol metabolism, most likely by producing mediators (e.g., PGE_2) that inhibit lipoprotein lipase, increase the supply of fatty acids to the liver, and increase generation of H_2O_2 via peroxisomal β-oxidation.

Based on sensitivity to the alcohol dehydrogenase (ADH) inhibitor 4-methylpyrazole, it was reported previously that ADH was involved in the swift increase in alcohol metabolism (SIAM) (Yuki and Thurman, 1980). This phenomenon is characterized by increased basal oxygen uptake, which provides NAD^+ for ADH-dependent alcohol metabolism (Yuki and Thurman, 1980). As glycogen reserves are depleted, glycolysis is slowed and ADP is shuttled into the mitochondria, where respiration is increased. More recently, however, it has been shown that 4-methylpyrazole also inhibits acyl CoA synthetase (Bradford et al., 1993a), a pivotal enzyme in the synthesis of acyl CoA compounds required for the generation of H_2O_2 via peroxisomal β-oxidation. Catalase is localized in the peroxisome, and the catalase pathway requires H_2O_2, which is provided largely by metabolism of fatty acids via peroxisomal β-oxidation (Lazarow and de Duve, 1976; Handler and Thurman, 1988). Methanol is a known selective substrate for catalase in rodents and is an excellent tool for

the evaluation of catalase-dependent alcohol metabolism without the use of inhibitors (Feytmans et al., 1974; Bradford et al., 1993a). It is well known that ethanol stimulates peripheral lipolysis and increases circulating triglycerides (Khanna et al., 1974); however, whether catalase participates in the mechanism of SIAM is not known.

It has been demonstrated that Kupffer cells, the resident hepatic macrophages, participate in the pathophysiology of ethanol-induced liver damage (Adachi et al., 1995). When Kupffer cells are activated, potent cytokines such as platelet-activating factor and tumor necrosis factor-α are released. In addition, Kupffer cells release prostaglandin E_2 (PGE_2), which stimulates oxygen uptake by parenchymal cells via increases in cAMP (Qu et al., 1996). $GdCl_3$ specifically destroys large Kupffer cells without causing other morphological changes in liver (Hardonk et al., 1992). Moreover, $GdCl_3$ diminishes inflammation and necrosis due to ethanol (Adachi et al., 1994) and fibrosis using model compounds (Sullivan et al., 1995; Wall et al., 1995). Furthermore, $GdCl_3$ treatment blocked the increase in hypoxia and production of α-hydroxyethyl radicals associated with chronic ethanol exposure

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ABBREVIATIONS: PGE_2, prostaglandin E_2; LPL, lipoprotein lipase; ADH, alcohol dehydrogenase; ANOVA, analysis of variance; SIAM, swift increase in alcohol metabolism.

(Adachi et al., 1994; Knecht et al., 1995). Therefore, this study will test the hypothesis that Kupffer cells and catalase are involved in the hypermetabolic state and the swift increase in alcohol metabolism (SIAM). Preliminary accounts of this work have appeared elsewhere (Bradford et al., 1994).

Materials and Methods

Treatment of Rats. Fed, female Sprague-Dawley rats (100–120 g) were used in this study. $GdCl_3$ (10 mg/kg) dissolved in acidic saline (pH 3.0) was injected into the tail vein 24 h before perfusion in some rats. This dose of $GdCl_3$ has been shown to remove large Kupffer cells without causing other morphological changes in the liver (Hardonk et al., 1992). Moreover, it eliminates about 80% of Kupffer cells based on mRNA for a specific Kupffer cell lectin (Koop et al., 1997). Ethanol (5.0 g/kg), methanol (5.0 g/kg), and olive oil (2 ml/100 g b.wt.), a good source of oleate, were administered intragastrically 2.5 h before liver perfusion. In some experiments, indomethacin (3.0 mg/kg, in dimethylsulfoxide) was administered intragastrically 1 h before ethanol.

Liver Perfusion and Alcohol Metabolism. Livers were perfused using hemoglobin-free Krebs-Henseleit buffer under conditions that were established more than 30 years ago (Scholz, 1968) and have been used for studies of hepatic metabolism, oxidation of xenobiotics, and metabolism of alcohols (reviewed in Brouwer and Thurman, 1996). The oxygen concentration in the effluent perfusate was monitored using a Teflon-shielded, Clark-type oxygen electrode. After oxygen uptake reached steady state values in about 15 min, the perfusion system was converted to a closed system with a 50-ml volume containing either 25 mM ethanol or methanol. Perfusate was reoxygenated using a Silastic tube oxygenator (Handler et al., 1986). Samples of perfusate (0.5 ml) were collected every 10 to 15 min, and the decrease in alcohol concentration over time was measured with head-space gas chromatography as described in detail elsewhere (Bradford et al., 1993a). Rates of alcohol metabolism were calculated based on changes in concentration over time and were expressed per gram of liver per hour. After perfusion, the liver was fixed with a solution of 5% buffered formalin and was perfused with alcohol for 15 min. Alcohol elimination was monitored during this period to correct for vaporization of alcohol from the organ surface, which was minimal.

Enzyme Measurements. Liver homogenates (1:10) were prepared in 0.25 M sucrose, and activities of catalase, acyl CoA synthetase, and acyl CoA oxidase were determined as described previously (Bradford et al., 1993a). Lipoprotein lipase (LPL) activity was determined in homogenized retroperitoneal fat pads as described elsewhere (Borensztajn et al., 1970). Measurement of LPL activity required chylomicrons that were harvested from fasted rats treated for 4 h with olive oil (2 ml/100 g b.wt. i.g.). Rats were anesthetized with pentobarbital (50 mg/kg) and chylomicrons (100–150 μEq of triglyceride fatty acid/ml) were collected from the thoracic duct for 2 h and frozen (−20°C) for subsequent use (Borensztajn et al., 1970). Rats were anesthetized, and retroperitoneal fat pads were harvested and homogenized in saline (1:40). Homogenates (56 μl) were incubated at 37°C for 2 h in a cocktail containing 2 volumes of albumin (20% w/v in water, pH 8.1), 1 volume of 0.7 M Tris·HCl (pH 8.1), and 0.5 volume each of serum, heparin (14 IU/ml), and chylomicrons to provide appropriate substrates for LPL. The reaction was stopped by adding 50 μl of incubation mixture to 250 μl of Dole's extraction mixture, and free fatty acids were extracted. Free fatty acids were washed with heptane, isolated, and determined colorimetrically (Novak, 1965). Triglycerides were determined in plasma using a spectrophotometric assay after hydrolysis to glycerol and free fatty acids (Bucolo and David, 1973).

PGE_2 from Kupffer Cells. Rats were treated with saline or ethanol (5.0 g/kg) and were killed 2 h later for isolation of Kupffer cells. Isolation was performed using collagenase digestion and differential centrifugation with Percoll as described previously (Pertoft

and Smedsrod, 1987). Primary cultures of Kupffer cells from control or ethanol treated rats were incubated for 4 h. Supernatants were assayed for PGE_2 by competitive radioimmunoassay using ^{125}I-labeled PGE_2 (Advanced Magnetics, Cambridge, MA).

Statistics. Statistical comparisons were made using analysis of variance (ANOVA) with Bonferroni's post hoc comparisons, Student's t test, or two-way ANOVA with Tukey's post hoc comparisons on ranks as appropriate. $p < .05$ was selected before the study as the level of significance.

Results

Oxygen Uptake and Alcohol Metabolism by Perfused Liver after Acute Exposure to Alcohol In Vivo (SIAM). Figure 1 (top) depicts typical liver perfusion experiments from a control animal and a rat 2.5 h after treatment with 5.0 g/kg methanol in vivo. In this experiment, basal rates of oxygen uptake, which were monitored continuously, were around 100 μmol/g/h and were increased to 200 μmol/g/h after methanol treatment in vivo. Values increased about
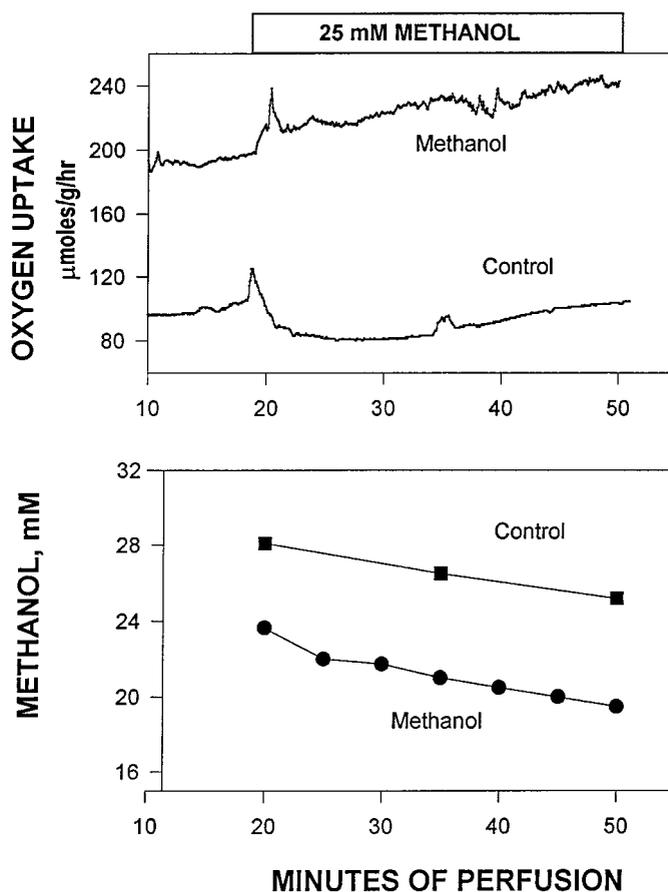

Fig. 1. Effect of methanol treatment in vivo on rates of oxygen and methanol uptake in the perfused liver. Top, oxygen uptake by an isolated perfused liver from a control rat or from a rat treated 2.5 h earlier with methanol in vivo (5.0 g/kg i.p.). The liver was perfused for 10 min using a nonrecirculating system with Krebs-Henseleit buffer (pH 7.4, 37°C), followed by 35 min with buffer containing 25 mM methanol in a recirculating system. Rates of oxygen uptake were determined with a Clark-type electrode. Bottom, methanol metabolism by the perfused liver. Samples of perfusate were collected and analyzed for methanol as described in *Materials and Methods*. Rates of methanol uptake were 23 μmol/g/h in the control and 67 μmol/g/h after methanol treatment determined by measuring the decrease in concentration and values calculated based on liver weight, volume of the perfusate, and time. Results are from a typical experiment repeated eight times.

20% after the addition of 25 mM methanol to the perfusate, most likely due to an increase in peroxisomal oxygen demand (see also Fig. 3, top). The methanol concentration in the perfusate decreased over time at a rate of 23 μmol/g/h in the control and 67 μmol/g/h after methanol treatment (Fig. 1, bottom). Figure 3 summarizes the effect of methanol treatment in vivo on rates of oxygen and methanol uptake by the perfused liver. Average rates of oxygen uptake by the perfused liver were increased significantly from 114 $\pm$ 12 to 192 $\pm$ 5 μmol/g/h by methanol treatment in vivo. Methanol uptake was likewise increased from 22 $\pm$ 6 to 83 $\pm$ 18 μmol/g/h. Thus, like ethanol, methanol, which is not a substrate for ADH in rodents, can produce a SIAM phenomenon.

Figure 2 summarizes the effect of GdCl$_3$ treatment and ethanol administration in vivo on oxygen uptake (top) and methanol metabolism (bottom) by the perfused liver. Basal rates of oxygen uptake were nearly doubled after treatment with ethanol 2.5 h before perfusion as expected: the "SIAM" phenomenon (Yuki and Thurman, 1980; Bradford et al., 1993b). Treatment with GdCl$_3$ did not alter basal rates of oxygen uptake, but the increase observed with ethanol treatment was blocked. Rates of methanol metabolism by the perfused liver increased from 22 $\pm$ 6 to 55 $\pm$ 10 μmol/g/h as a result of 2 to 3 h of ethanol treatment in vivo. It has been demonstrated that when Kupffer cells were destroyed with GdCl$_3$, the hypermetabolic state due to ethanol treatment in

vivo was blocked (Bradford et al., 1993b). In the current study, GdCl$_3$ treatment also blocked the increase in methanol metabolism due to alcohol treatment (Fig. 2, bottom). Furthermore, the cyclooxygenase inhibitor indomethacin completely blocked the stimulation of oxygen uptake by ethanol. In these experiments, stimulated rates of oxygen uptake (189 $\pm$ 18 μmol/g/h) due to ethanol were blunted by indomethacin (119 $\pm$ 15 μmol/g/h, $p < .03$), supporting the hypothesis that eicosanoids are involved in the stimulation of hepatic oxygen uptake after alcohol administration. Primary cultures of Kupffer cells were isolated 2 h after saline or ethanol treatment in vivo, and PGE$_2$ levels were increased significantly from 47 $\pm$ 6 (control) to 144 $\pm$ 31 (ethanol; $p < .006$) pmol/10^6 cells/4 h.

Effect of Acute Alcohol Treatment on Ketogenesis. Because free fatty acids could provide substrate for catalase-dependent alcohol metabolism, rates of ketogenesis were calculated from ketone body release into the effluent perfusate. Basal rates of ketogenesis were low and not different in those livers from control or GdCl$_3$-treated rats (Table 1); however, there was a significant, nearly 2-fold increase after acute ethanol treatment in this study, confirming earlier work (Yuki and Thurman, 1980). Moreover, this increase in ketone body production due to ethanol was blocked by GdCl$_3$ treatment (Table 1). Because peroxisomal fatty acid metabolism generates H$_2$O$_2$ for metabolism of alcohols via catalase, it is possible that GdCl$_3$ blocks the production of H$_2$O$_2$ when excess fatty acid is present. To test this hypothesis, rats were given olive oil, a good source of oleate, to provide exogenous fatty acids in vivo for H$_2$O$_2$ production 2.5 h before perfusion (Fig. 3). Under these conditions, rates of oxygen uptake were elevated significantly from 114 $\pm$ 13 to 156 $\pm$ 14 μmol/g/h, a phenomenon that was unaffected by GdCl$_3$. Methanol metabolism was also increased about 3-fold by the addition of exogenous fatty acids, a phenomenon that was also not affected by GdCl$_3$.

Fatty Acid Supply. Because peroxisomal β-oxidation requires fatty acids, plasma triglycerides and several key enzymes involved in lipid metabolism (e.g., LPL and acyl CoA synthetase) were measured. LPL activity in retroperitoneal fat pads was decreased significantly by about 60% after alcohol treatment, an effect blocked by GdCl$_3$ (Table 2). Plasma triglycerides were increased nearly 2-fold by ethanol treatment as expected, an effect also blocked by treatment with GdCl$_3$ (Table 2). Hepatic acyl CoA synthetase activity was increased slightly but significantly 2.5 h after ethanol
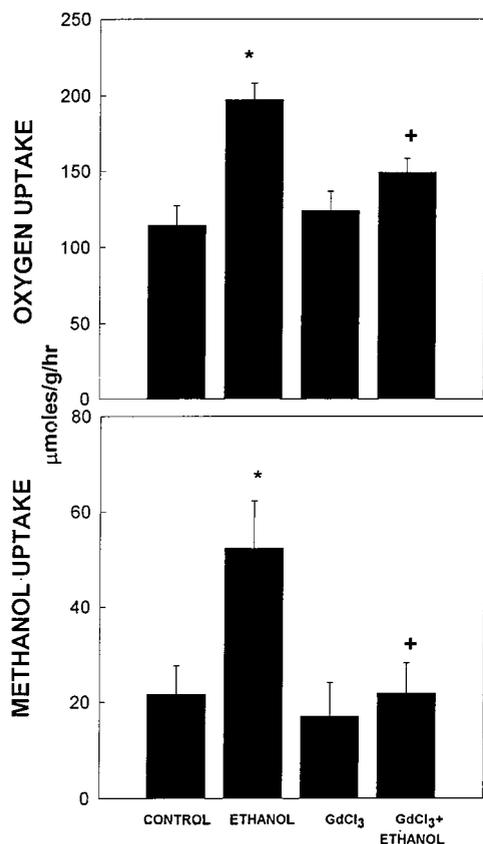

Fig. 2. Effect of ethanol treatment in vivo on rates of oxygen and methanol uptake by the perfused liver. Rats were treated with GdCl$_3$ and/or ethanol in vivo as described in *Materials and Methods*, and livers were perfused as described in Fig. 1. Statistical comparisons were made with the control group using ANOVA with Bonferroni's post hoc comparisons. Values are mean $\pm$ S.E.M. from five to nine livers per group. *$p < .05$, for comparisons with control. +$p < .05$ for comparisons with ethanol group.

TABLE 1

Effect of acute ethanol treatment and GdCl$_3$ in vivo on ketogenesis in the perfused liver

Ethanol or saline was given i.g. 2.5 h before perfusion to fed rats after treatment in vivo with 10 mg/kg GdCl$_3$ or saline i.v. 24 h before perfusion as described in the text. Livers were perfused for 20 min, and acetoacetate (A) and β-hydroxybutryrate (B) were determined enyzmatically in samples of effluent perfusate (Bergmeyer, 1988). Results are expressed as mean $\pm$ S.E.M., $n = 5$–7. Statistical comparisons were made using two-way ANOVA on ranks and Tukey's post hoc test.

Treatment	Ketogenesis β-Hydroxybutryrate + Acetoacetate
	$\mu mol/g/h$
Control	11.4 $\pm$ 0.4
Ethanol	27.7 $\pm$ 5.5[a]
GdCl$_3$	12.5 $\pm$ 1.7
GdCl$_3$ + ethanol	12.3 $\pm$ 4.5[b]

[a] $p < .05$ for comparison with the control group.
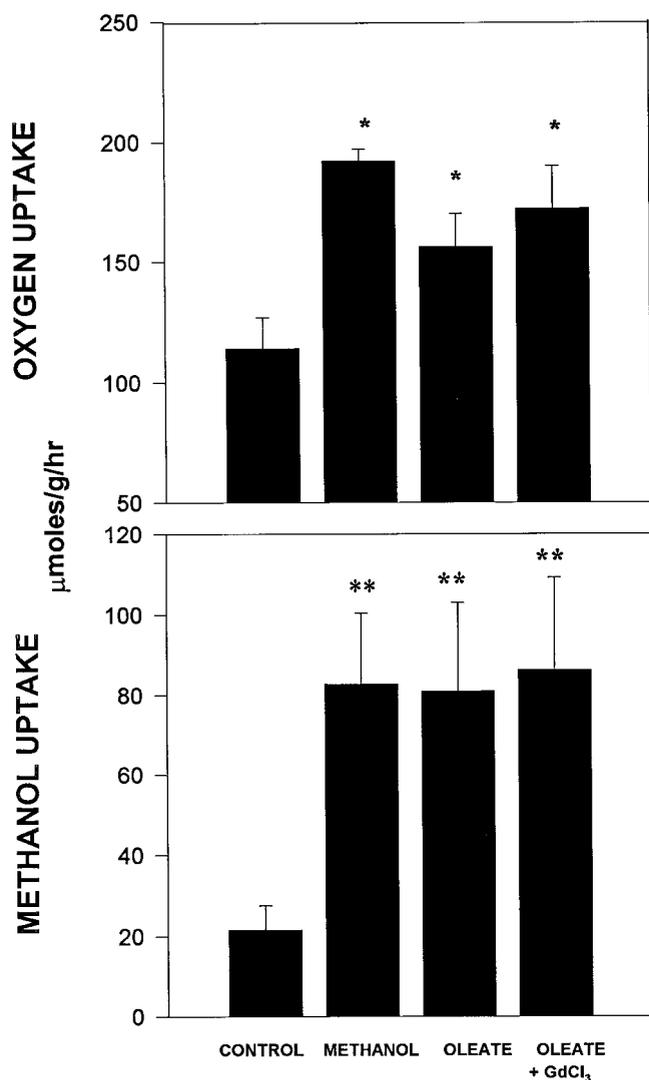[b] $p < .05$ for comparison with the ethanol group.

Fig. 3. Effect of methanol and oleate treatment in vivo on oxygen and methanol uptake by the perfused liver. Rats were treated with methanol or olive oil in vivo, and livers were perfused as described in *Materials and Methods*. GdCl₃ was administered to some rats as described in *Materials and Methods*. Statistical comparisons were made using ANOVA with Bonferroni's post hoc comparisons. Values are mean ± S.E.M. from five to eight livers per group, $*p < .05$ for comparison with the control group.

TABLE 2
Effect of ethanol and GdCl₃ on triglycerides, LPL, and acyl CoA synthetase

Fed rats were treated in vivo as described in the text and in the legend to Table 1. Rats were killed, and retroperitoneal fat pads were removed and homogenized. Blood from the inferior vena cava was collected, diluted 1:10 with sodium citrate (3.8%), and centrifuged to obtain plasma. Triglycerides, LPL, and acyl CoA synthetase were determined as described in the text. Results are expressed as mean ± S.E.M., $n = 4$–16. Statistical comparisons were made using ANOVA with Bonferroni's post hoc comparisons.

Treatment	Plasma Triglycerides	Fat Pad LPL	Liver Acyl CoA Synthetase
	mg/dl	*U/g tissue*	*nmol/mg protein/min*
Control	19.1 ± 1.6	76.0 ± 14.9	14.1 ± 1.0
Ethanol	37.4 ± 7.2[a]	23.7 ± 8.5[a]	17.9 ± 0.7[a]
GdCl₃ + ethanol	21.8 ± 4.2	52.5 ± 21.7	16.4 ± 1.2

[a] $p < .05$ for comparison with the control group.

treatment (Table 2), and this effect was also blocked by GdCl₃. On the other hand, catalase was unaltered by GdCl₃ (control, 2891 ± 367 U/g liver; GdCl₃, 2781 ± 180 U/g liver),

and acyl CoA oxidase remained unchanged (control, 0.8 ± 0.3 nmol/mg protein/min; GdCl₃, 1.1 ± 0.6 nmol/mg protein/min).

Discussion

The Catalase Pathway Is Activated Rapidly by Both Ethanol and Methanol. When ethanol or methanol was given in vivo, rates of methanol metabolism increased significantly in only a few hours, leading to the conclusion that the catalase pathway is stimulated by alcohols (Figs. 2 and 3).

It has been demonstrated that catalase-dependent alcohol metabolism is regulated by H₂O₂ supply (Oshino et al., 1973), which arises predominately from fatty acid oxidation by peroxisomes (Fig. 4) (Handler and Thurman, 1985). Rates of methanol metabolism were stimulated after treatment with oleate, and values were not altered by the destruction of Kupffer cells, suggesting that these cells do not effect transport of CoA compounds into the peroxisome. Additionally, catalase and acyl CoA oxidase were unchanged by destruction of Kupffer cells with GdCl₃ (see *Results*). On the other hand, acute ethanol treatment in vivo elevates triglycerides in plasma (Table 2 and *Results*) due partly to stimulation of lipases by adrenergic hormones and a decrease in retroperitoneal LPL activity (Table 2) (Brodie et al., 1961; Elko et al., 1961). Moreover, increased rates of ketogenesis demonstrate that utilization of fatty acids after alcohol is elevated (Table 1).

In a recent study, it was demonstrated that an acute dose of methanol in vivo significantly elevated fatty acid methyl ester levels in liver (Kaphalia et al., 1995) and increased levels of palmitic, stearic, linoleic, oleic, and arachidonic acids within 3 h. When methanol was given 2.5 h before perfusion here, rates of oxygen uptake and methanol metabolism were stimulated significantly, consistent with the hypothesis that increased levels of long-chain fatty acids provide substrate for production of H₂O₂ necessary for catalase-dependent alcohol metabolism (Fig. 3). It has previously been demonstrated that rates of methanol metabolism by the
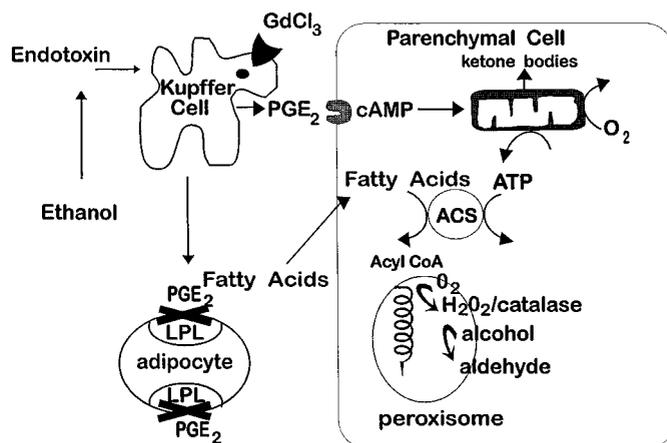

Fig. 4. Working hypothesis for the involvement of peroxisomal fatty acids in SIAM. Alcohol increases plasma endotoxin, which activates Kupffer cells to produce mediators such as PGE₂ that inhibit LPL and elevate free fatty acids. PGE₂ elevates cAMP, which is involved in the hypermetabolic state caused by ethanol. Adrenergic stimulation by alcohol also increases peripheral lipolysis and increases triglycerides in blood. Fatty acids are metabolized, and fatty acyl CoA oxidase generates H₂O₂ for catalase-dependent alcohol metabolism in the peroxisomes. Importantly, these biochemical events are blocked if Kupffer cells are destroyed by GdCl₃.

perfused liver can be elevated significantly when fatty acids are infused (Handler and Thurman, 1987). Here, catalase-dependent methanol metabolism in perfused liver was increased from 16 to 67 μmol/g/h by oleate, values that were inhibited completely with the catalase inhibitor aminotriazole (Handler and Thurman, 1987). Under these conditions, ethanol metabolism was diminished about 70%. When aminotriazole was given before ethanol or methanol in deer mice lacking ADH, rates of ethanol and methanol metabolism were diminished nearly completely in vivo (Bradford et al., 1993c). Thus, aminotriazole effectively inhibits catalase-dependent methanol and ethanol metabolism in vivo and in perfused liver. In this study, when oleate was administered alone, providing an excess of fatty acid, oxygen and methanol metabolisms were both stimulated dramatically (Fig. 3). It is concluded that this phenomenon is Kupffer cell independent because it was GdCl$_3$ insensitive (Fig. 3). Taken together, it is concluded that Kupffer cells participate in regulation of H$_2$O$_2$ supply via increasing delivery of lipid to peroxisomal β-oxidation in the liver (see below).

PGE$_2$ Participates in SIAM by Providing Lipid. SIAM requires activation of oxygen uptake and cofactor supply for alcohol metabolism. Is there evidence to support the hypothesis that PGE$_2$ plays a role in SIAM? A link between Kupffer cells and regulation of oxygen uptake was made recently. Qu et al. (1996) demonstrated that Kupffer cells produce PGE$_2$ in sufficient quantities to stimulate respiration of isolated parenchymal cells. Media from cultured Kupffer cells isolated from rats fed ethanol chronically stimulated respiration about 30% in parenchymal cells. In this study, PGE$_2$ was significantly higher in media from Kupffer cells isolated from rats after acute ethanol treatment (see *Results*). Furthermore, the stimulation of oxygen uptake was blocked by the cyclooxygenase inhibitor indomethacin (see *Results*) (Qu et al., 1996). This study demonstrated that activation of oxygen uptake in the perfused liver by alcohol is dependent on mediators such as PGE$_2$ from Kupffer cells.

Several studies have examined the interactions between prostaglandins and fatty acid supply (Feingold et al., 1992; Hardardottir et al., 1992; Flisiak et al., 1993). Regulation of lipolysis has been linked to prostaglandin synthesis (Feingold et al., 1992), and a recent study demonstrated that LPL gene expression in peritoneal macrophages was inhibited by PGE$_2$ (Desanctis et al., 1994). Additionally, indomethacin was shown to overcome the effects of endotoxin on LPL inhibition (Desanctis et al., 1994) and blocked the increase in PGE$_2$ due to ethanol in stellate cells (Flisiak et al., 1993). In this study, ethanol stimulated PGE$_2$ release from Kupffer cells and decreased LPL activity (Table 2). This causes an increase in free fatty acids, which are required substrates for catalase-dependent alcohol metabolism. This phenomenon was blocked when Kupffer cells were destroyed with GdCl$_3$ as well as ethanol-induced changes in plasma triglycerides and acyl CoA synthetase (Table 2). Thus, it is concluded that PGE$_2$ from the Kupffer cell plays a pivotal role in the supply of free fatty acids to the liver (Fig. 4).

Taken together, these data clearly support the hypothesis that Kupffer cells are involved in SIAM. The scheme depicted in Fig. 4 summarizes the key elements of this working hypothesis. It is hypothesized that alcohol increases plasma endotoxin (Enomoto et al., 1998), which activates Kupffer cells to produce mediators such as PGE$_2$ that inhibit periph-

eral LPL, resulting in an increase in free fatty acids and activation of mitochondrial respiration via cAMP (Hassid, 1986; Garcia et al., 1997). Adrenergic stimulation by alcohol also contributes to increased ketone body formation and triglycerides in the blood. Fatty acyl CoA oxidase generates H$_2$O$_2$ for catalase-dependent alcohol metabolism from free fatty acids in the peroxisome. Importantly, ethanol-induced increases in LPL activity, acyl CoA synthetase activity, ketone body formation, and plasma triglycerides returned to normal levels when Kupffer cells were destroyed.

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Send reprint requests to: Dr. Ronald G. Thurman, Doctor of Philosophy, Department of Pharmacology, Laboratory of Hepatobiology and Toxicology, CB #7365 Mary Ellen Jones Building, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7365. E-mail: thurman@med.unc.edu.

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