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Mitochondrial remodeling in the liver following chronic alcohol feeding to rats

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Abstract

The feeding of alcohol orally (Lieber-DeCarli diet) to rats has been shown to cause declines in mitochondrial respiration (state III), decreased expression of respiratory complexes, and decreased respiratory control ratios (RCR) in liver mitochondria. These declines and other mitochondrial alterations have led to the hypothesis that alcohol feeding causes “mitochondrial dysfunction” in the liver. If oral alcohol feeding leads to mitochondrial dysfunction, one would predict that increasing alcohol delivery by intragastric (IG) alcohol feeding to rats would cause greater declines in mitochondrial bioenergetics in the liver. In this study, we examined the mitochondrial alterations that occur in rats fed alcohol both orally and intragastrically. Oral alcohol feeding decreased glutamate/malate-, acetaldehyde- and succinate-driven state III respiration, RCR, and expression of respiratory complexes (I, III, IV, V) in liver mitochondria, in agreement with previous results. IG alcohol feeding, on the other hand, caused a slight increase in glutamate/malate-driven respiration, and significantly increased acetaldehyde-driven respiration in liver mitochondria. IG feeding also caused liver mitochondria to experience a decline in succinate-driven respiration, but these decreases were smaller than those observed with oral alcohol feeding.

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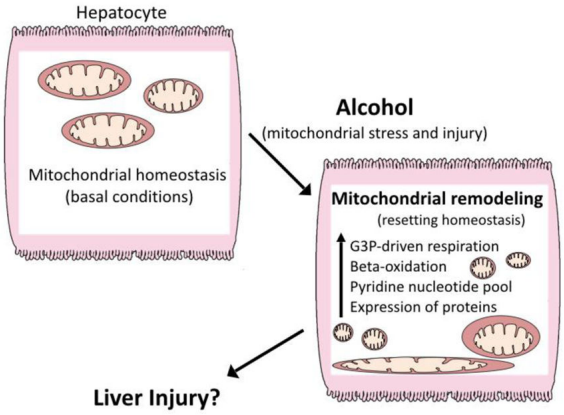
Surprisingly, oral and IG alcohol feeding to rats increased mitochondrial respiration using other substrates, including glycerol-3-phosphate (which delivers electrons from cytoplasmic NADH to mitochondria) and octanoate (a substrate for beta-oxidation). The enhancement of glycerol-3-phosphate- and octanoate-driven respiration suggests that liver mitochondria remodeled in response to alcohol feeding. In support of this notion, we observed IG alcohol feeding also increased expression of mitochondrial glycerol phosphate dehydrogenase-2 (GPD2), transcription factor A (TFAM), and increased mitochondrial NAD^+-NADH and $NADP^+$-NADPH levels in the liver. Our findings suggest that mitochondrial dysfunction represents an incomplete picture of mitochondrial dynamics that occur in the liver following alcohol feeding. While alcohol feeding causes some mitochondrial dysfunction (i.e. succinate-driven respiration), our work suggests that the major consequence of alcohol feeding is mitochondrial remodeling in the liver as an adaptation. This mitochondrial remodeling may play an important role in the enhanced alcohol metabolism and other adaptations in the liver that develop with alcohol intake.

Graphical abstract



Keywords

mitochondria; respiration; alcohol; liver; remodeling

Introduction

Mitochondrial remodeling and biogenesis may be an important mechanism in the adaptation of cells to stress and metabolic changes [1]. In skeletal muscle cells, chronic exercise has been shown to cause mitochondrial biogenesis and remodeling, involving increased expression of respiratory complexes and mitochondrial proteins involved in β-oxidation [2-4]. These mitochondrial alterations are believed to enhance mitochondrial respiration and β-oxidation to increase energy production as an adaptation to exercise. Metabolic changes, such as increased fatty acid intake, have also been shown to increase mitochondrial biogenesis and respiration in muscle cells to increase β-oxidation capacity [5]. In another form of mitochondrial remodeling, cell starvation has been shown to alter mitochondrial fusion-fission to produce elongated mitochondria [6]. Morphological changes, such as elongation, can enhance mitochondrial respiration by increasing cristae surface area, and

increase mitochondrial half-life by decreasing mitophagy. Mitochondrial remodeling, or plasticity, is therefore an important mechanism underlying cellular adaptation to many types of stresses and metabolic changes [1].

We have previously shown that chronic alcohol feeding to mice resulted in mitochondrial remodeling involved in the adaptation of the liver to alcohol [7]. Intragastric alcohol (IG) feeding, a method that allows for delivery of high levels of alcohol, led to ~2-fold enhancement of mitochondrial respiration in mouse liver. This enhanced mitochondrial respiration may be due to increased expression of respiratory complex proteins, increased levels of mitochondrial NAD^+-NADH, and possibly be due to alterations in mitochondrial morphology. The increased liver mitochondrial respiration caused by alcohol feeding was associated with increased acetaldehyde metabolism, likely due to enhanced NAD^+ regeneration by the electron transport chain. NAD^+ is the rate-limiting substrate needed by alcohol dehydrogenase (ADH) for alcohol metabolism and aldehyde dehydrogenase 2 (ALDH2) for acetaldehyde metabolism (reaction 1 and 2) [8, 9].

$$\text{Ethanol} + \text{NAD}^+ \rightarrow \text{acetaldehyde} + \text{NADH} + \text{H}^+ \quad \text{(1) catalyzed by ADH}$$

$$\text{Acetaldehyde} + \text{NAD}^+ + \text{H}_2\text{O} \rightarrow \text{acetate} + \text{NADH} + \text{H}^+ \quad \text{(2)}$$

catalyzed by ALDH2

Therefore, enhanced mitochondrial respiration may be an important adaptation to alcohol in the liver to enhance alcohol metabolism by increasing NAD^+ regeneration. Oral alcohol feeding to mice (Lieber-DeCarli model), which delivers lower doses of alcohol, caused much less mitochondrial remodeling than IG alcohol feeding. Other studies on mice that were fed alcohol orally have also observed some evidence of mitochondrial remodeling, including higher levels of complex I and increased mitochondrial respiration [10, 11].

Our findings appear to challenge the established dogma that alcoholic liver disease primarily involves mitochondrial dysfunction [12-14]. The mitochondrial dysfunction hypothesis is primarily based on studies involving oral alcohol feeding to rats (Lieber-DeCarli diet), which has been shown to cause a decline in mitochondrial respiration (state III) and a decline in the respiratory control ratio (RCR) in liver mitochondria [15-17]. The decline in mitochondrial respiration in liver was accompanied by a decline in ribosome activity and a decline in the synthesis of respiratory complex proteins in liver of rats fed alcohol orally [14, 18]. These findings contradict mice studies showing increased mitochondrial bioenergetics with alcohol feeding, suggesting that liver mitochondria in rats and mice may respond very differently to alcohol. However, a detailed investigation of mitochondrial changes that occur in rats with high alcohol feeding, which can be achieved by IG alcohol feeding [19, 20], has not been performed. If alcohol feeding leads to mitochondrial dysfunction in rats, one would

predict that increased alcohol delivery by IG feeding should cause a greater decline in mitochondrial bioenergetics in the liver.

In humans, there is evidence that chronic alcohol use causes mitochondrial dysfunction in late stages of alcoholic liver disease [21]. However, there is also some evidence of mitochondrial remodeling, with upregulation of mitochondrial enzymes, such as glutamate dehydrogenase in the liver of alcoholic patients [22]. In this study, we examine the mitochondrial alterations that occur in rats fed alcohol both orally and intragastrically. A complete analysis of often overlooked mitochondrial bioenergetics parameters, such as glycerol-3-phosphate-driven respiration in rats fed alcohol was performed. The question of whether alcohol feeding in rats causes mitochondrial dysfunction, mitochondrial remodeling, or both is examined in this work.

Material and Methods

Animals

Wistar rats (150 g) were obtained from Charles Rivers (Wilmington, MA). The animals were housed in a temperature-controlled room and were acclimatized for a minimum of 3 days prior to use in experiments. All animals received care according to methods approved under institutional guidelines for the care and use of laboratory animals in research.

Oral alcohol feeding to mice and rats—Rats were fed a commercially available liquid diet (Bioserve, NJ) to which ethanol was mixed at 5.4% (w/v) via a feeding tube. Pair feeding was done to a control animal by feeding the equal amount consumed by an ethanol-fed rat except that ethanol was isocalorically replaced with dextrin. Rats were fed alcohol orally for 6 weeks.

IG alcohol feeding—IG alcohol fed rats and control rats were provided by the Southern California Research Center for ALPD and Cirrhosis. Rats were implanted with a long-term gastrostomy catheters for alcohol infusion as previously described [19]. Briefly, after 1 week of acclimatization, rats were infused with a control high fat diet with or without alcohol. Alcohol infusion was initiated at a dose of 22.7 g/kg/day, and gradually increased. Alcohol accounted for ~ 32.9% of total caloric intake after 1 week of IG alcohol feeding. By 4 weeks of IG alcohol feeding, the caloric intake of alcohol accounted for ~ 38.4% of total caloric intake. Rats were fed alcohol intragastrically for 6 weeks.

Biochemical assays

Isolation of liver mitochondria—Liver mitochondria from alcohol fed rats were isolated using differential centrifugation as previously described [23]. Livers were excised, washed with 0.25 M sucrose and homogenized in an H-medium (210 mM mannitol, 70 mM sucrose, 2 mM HEPES, 0.05% bovine serum albumin (w/v), plus protease and phosphatase inhibitors). The homogenate was centrifuged at 850 g for 10 minutes, the pellet (cellular debris) was removed, and the centrifugation process was repeated. The resulting supernatant (cytoplasmic fraction) was centrifuged at 8,500 g for 15 min. The pellet, which represents the mitochondrial fraction, was washed with H-medium and the centrifugation was repeated.

The mitochondria were resuspended in H-medium before oxygen electrode and Western blot analyses. Immunoblotting showed relatively pure fractions, as little actin (cytoplasmic protein) was found in the mitochondrial fraction and little glutamate dehydrogenase (GLUD; mitochondrial protein) was observed in the cytoplasmic fraction (Fig 9).

Measurements of respiration in isolated mitochondria—Respiration was measured in freshly isolated mitochondria by monitoring oxygen consumption with a Clark-type electrode (Hanstech, UK) in respiration buffer containing 230 mM mannitol, 70 mM sucrose, 30 mM Tris-HCl, 5 mM KH_2PO_4, 1 mM EDTA, pH 7.4 [23]. Isolated mitochondria (0.50-0.70 mg) were added to 1ml of respiration buffer and oxygen consumption monitored in the presence of mitochondrial substrates (glutamate/malate 7.5 mM – complex I substrates; succinate 7.5 mM – complex II substrates) with or without ADP (250 M). In some experiments acetaldehyde (250 uM) was used as a substrate (complex I) for mitochondrial respiration measurements. Mitochondrial respiration was also measured using glycerol 3-phosphate (2.5 mM), which feeds into glycerol phosphate dehydrogenase-2 in the mitochondrial inner membrane, and octanoate (200 uM), a medium chain fatty acid that undergoes beta-oxidation. State IV respiration is defined as respiration in the presence of substrates, while state III respiration is defined as respiration in the presence of both substrates and ADP. The RCR is defined as state III/state IV.

Immunoblotting—Aliquots of cytoplasmic or mitochondrial extracts were fractionated by electrophoresis on 8-12% SDS polyacrylamide gels (Biorad, Hercules, CA). Subsequently, proteins were transferred to nitrocellulose or PVDF membranes and blots were blocked with 5% (w/v) nonfat milk dissolved in Tris-buffered saline (TBS) with Tween-20. Complex I (NDUFS3 subunit), II (SDHA subunit), complex III (subunit 1), V (a subunit), and MCAD antibodies were obtained from Mitosciences (Eugene, OR). Complex IV, actin, and acetylation antibodies were obtained from Cell Signaling Technology (Danvers, MA). The antibody to GLUD was obtained from Santa Cruz (Santa Cruz, CA), the antibody to HNE was obtained from Abcam (Cambridge, MA), and the TFAM antibody was obtained from Avia Systems Biology (San Diego, CA). Mitochondrial glycerol phosphate dehydrogenase-2 (GPD2) antibody was obtained from Proteintech (Chicago, Il). In rat liver mitochondria GPD2 was found primarily as a dimer (~ 136 kD), instead of the monomer (~ 68 kD). SDS treatment (4X) was used to convert a large percent of dimer to monomer form, but still some dimer form remained. In mouse mitochondria, GPD2 was primarily found in the monomer form. All blots shown are representative samples from 3-7 experiments. Densitometry was performed using the Image J software from NIH and normalized with appropriate loading controls.

HPLC measurement of pyridine nucleotides—$NADP^+$, NADPH, NAD^+, and NADH levels were measured by HPLC, as previously described in IG and oral alcohol fed rats [24]. Briefly, liver homogenate and isolated mitochondria were homogenized in buffer (0.06 M KOH, 0.2 M KCN and 1 mM bathophenanthroline disulfonic acid) followed by chloroform extraction. Chloroform extraction was carried out by centrifugation at 14,000 rpm in a microcentrifuge at 4°C; the resulting aqueous supernatant with soluble pyridine nucleotides was collected and extracted thrice to remove lipids and proteins. Finally, it was filtered with

a 0.45 μm positively charged filter (Pall Life Sciences) to remove RNA and DNA in a microcentrifuge at 4°C. The mobile phase consisted of 0.2 M ammonium acetate (buffer A) at pH 5.5, and HPLC-grade methanol (buffer B). A gradient program with initial conditions as 100% buffer A and 0% buffer B was set. From 0-4 min, 0-3% B and from 4-23 min, 3-6.8 % B, followed by washing the column with 50% A and 50% B and re-equilibrated to initial conditions for the next run. Quantitation of pyridine nucleotides was performed by integrating the peaks and adding the cyanide adducts as detected by the fluorescence spectrophotometer (exc = 330 nm; em = 460 nm).

Statistical analysis—Statistical analyses were performed using the Student's t test for unpaired data or ANOVA for comparison of multiple groups. $P < 0.05$ was defined as statistically significant.

Results

Oral and IG alcohol feeding promotes liver injury and hepatomegaly in rats

Both oral and IG alcohol feeding significantly increased serum alanine aminotransferase (ALT), indicating greater liver injury, and significantly induced hepatomegaly (greater liver weight, liver/body weight) compared to pair fed controls (Table 1). However, IG alcohol feeding, which delivers greater alcohol content, had significantly greater serum ALT levels (38%) than oral alcohol feeding to rats (Table 1). The differences in serum ALT levels between oral and IG alcohol fed rats were not as pronounced as differences observed in mice models, where increases in ALT levels are 3-4 fold greater with IG alcohol feeding [7]. Rats are in general believed to be more resistant to alcoholic liver injury [25]. IG alcohol feeding also caused greater hepatomegaly than oral alcohol feeding to rats, as there were significant differences in liver weight and the liver/body weight ratio between the two alcohol models (Table 1).

Alterations in mitochondrial state III respiration and respiratory control ratio in liver mitochondria from oral and IG alcohol fed rats

Mitochondrial respiration can be assessed by introducing various respiratory substrates that enter the electron transport chain at different sites (Fig 1). Glutamate/malate, which generates NADH that feeds into complex I, and succinate, which feeds into complex II, have been the most examined respiratory substrates in alcohol research. We observed that isolated liver mitochondria from rats fed alcohol orally exhibit a decline in glutamate/malate-driven and succinate-driven state III respiration compared to pair-fed control rats (Fig 2A), in agreement with previous results [15-17]. The decline in state III respiration corresponded with a decline in the respiratory control ratio (RCR; state III/state IV), which was significant using glutamate/malate as substrates (Fig 2B), also in agreement with previous findings [15]. IG alcohol feeding to rats, which delivers greater amounts of alcohol and causes greater liver injury (Table 1), did not cause a decline in glutamate/malate-driven state III respiration (Fig 2C), as observed following oral alcohol feeding. Succinate-driven respiration was reduced in liver mitochondria from rats fed alcohol intragastrically, though to a lesser extent than that in liver mitochondria from rats fed alcohol orally (IG 24%, oral 39%). IG alcohol feeding caused no significant declines in the RCR of isolated liver mitochondria (Fig 2D). These

findings show mitochondrial respiration were not worsen with greater alcohol dosing by IG feeding. On the contrary, oral alcohol feeding induced more declines in various mitochondrial parameters in the liver than IG feeding.

Remodeling of the respiratory complexes following oral and IG alcohol feeding to rats

Oral alcohol feeding to rats has been shown to decrease the respiratory complexes (I, III, IV, and V) in liver mitochondria using blue native gel electrophoresis (BN-PAGE) [26]. Utilizing immunoblotting of key respiratory complex proteins, we similarly observed a decline in complex I, III, IV, and V (Fig 3A). Although immunoblotting only examines one subunit of the complexes, there appears to be a strong correlation between our immunoblotting data and published BN-PAGE data. Interestingly, complex II expression was not suppressed, even though succinate-driven respiration was the most inhibited by oral alcohol feeding. IG alcohol feeding, on the other hand, caused a significant increase in complex II levels (Fig 3B; ~29%), even though succinate-driven respiration was inhibited with IG alcohol feeding to rats (Fig 2C). IG alcohol feeding also caused levels of complex I and III to significantly decline, although not to the same extent as oral alcohol feeding (oral I - 53%, III - 46% decreased; IG I - 30%, III - 31% decreased). In addition, complex IV and complex V levels did not significantly change with IG alcohol feeding, in contrast to oral alcohol feeding. These findings demonstrate that oral alcohol feeding in rats suppressed the expression of respiratory complex proteins in liver mitochondria more extensively than IG alcohol feeding. These findings also show that expression of respiratory complexes and respiration do not necessarily correlate, as succinate-driven respiration was the most inhibited, despite its levels remaining unchanged or increasing with alcohol feeding.

Effect of chronic alcohol feeding on acetaldehyde-, glycerol-3-phosphate-, and octanoate-driven mitochondrial respiration in isolated liver mitochondria

Acetaldehyde, the metabolite of alcohol metabolism generated by ADH, can act as a mitochondrial substrate, since ALDH2 generates NADH that feeds into complex I of the respiratory chain [27], similar to glutamate/malate (Fig 1). Previous studies have shown that acetaldehyde metabolism is limited by the rate of NAD^+ regeneration in the electron transport chain and thus, is coupled to mitochondrial respiration [7]. It was therefore surprising that acetaldehyde driven-respiration was inhibited in liver mitochondria following oral alcohol feeding [27]. In agreement with these findings, we observed that oral alcohol feeding to rats caused a significant decrease in state III respiration (~23%) in isolated liver mitochondria (Fig 4A). IG alcohol feeding to rats, on the other hand, significantly increased acetaldehyde-driven respiration (~37%) in isolated liver mitochondria (Fig 4A). Both oral and IG alcohol feeding did not significantly alter the RCR (Fig 4B). These findings suggest that respiration through complex I (glutamate/malate, acetaldehyde) is affected very differently by oral and IG feeding.

We next examined mitochondrial respiration using substrates that bypass complex I or II. Acyl-CoA dehydrogenases are key enzymes involved in beta-oxidation that feed electrons into the respiratory chain through electron-transferring flavoprotein (ETF) and ETF-ubiquinone oxidoreductase (Fig 1). We examined mitochondrial respiration utilizing octanoate, a medium chain fatty acid, that undergoes beta-oxidation in mitochondria through

the action of medium-chain acyl-coenzyme A dehydrogenase (MCAD). Octanoate-driven respiration was significantly increased in isolated liver mitochondria from both oral and IG alcohol fed rats (~ 33% for both; Fig 4C). The glycerol phosphate shuttle transfers electrons from NADH generated in the cytoplasm into the electron transport chain. In the glycerol phosphate shuttle, cytoplasmic glycerol phosphate dehydrogenase-1 (GPD1) transfers electrons from NADH to dihydroxyacetone phosphate to form glycerol-3-phosphate (G3P), which then shuttles electrons into the respiratory chain through the action of mitochondrial glycerol phosphate dehydrogenase-2 (GPD2) located in the inner membrane [28]. Thus, G3P is a respiratory substrate for GPD2 (Fig 1). As observed with octanoate, G3P-driven respiration was significantly enhanced in isolated liver mitochondria from both oral and IG alcohol fed rats (~26% oral; ~41% IG; Fig 4C). Utilizing octanoate and G3P, we observed for the first time that mitochondrial state III respiration can increase in the liver following oral alcohol feeding to rats. Changes in RCR were not statistically significant for both octanoate- and G3P-driven respiration, although in all case there was a decline with alcohol feeding (Fig 4D).

Remodeling of the mitochondrial proteins following oral and IG alcohol feeding to rats

We next examined protein levels by immunoblot analysis of key proteins involved in mitochondrial respiration driven by the respiratory substrates examined in Figure 4. Protein levels of ALDH2 and MCAD, which are important in shuttling electrons from acetaldehyde and octanoate, respectively, were not significantly altered with oral or IG alcohol feeding (Fig 5A and B). Protein levels of GPD2, on the other hand, were increased significantly in liver mitochondria from rats fed alcohol intragastrically (~33%; Fig 5B). Oral alcohol feeding did not cause a significant increase in GPD2 protein levels, although G3P-driven respiration was increased. GPD2 was expressed in rat liver mitochondria as both a dimer and a monomer, while in mice it was mainly seen in its monomeric form (Fig 6C). Both oral and IG alcohol feeding to rats also enhanced expression of mitochondrial transcription factor A (TFAM), a key transcription factor (nuclear gene) that is important for transcribing genes from mtDNA [29] (Fig 5). Increased TFAM expression suggests that transcription in mitochondria may increase with alcohol feeding. Overall, the fact that alcohol feeding, particularly IG alcohol feeding, caused an increase in several respiratory proteins suggests that mitochondrial remodeling occurred in the liver of rats in response to alcohol feeding.

Enhanced beta-oxidation and G3P-driven respiration also occurs in mice fed alcohol intragastrically

Since GPD2 plays a key role in transferring electrons generated by ADH in the cytoplasm to mitochondria, we explored whether this pathway was also enhanced in mice fed alcohol intragastrically. In mice, both octanoate- and G3P-driven respiration was enhanced by alcohol feeding (Fig 6A). As observed in rats, no statistically significant changes in RCR were observed, although in all case RCR tended to decline (Fig 6B). Protein levels of both MCAD and GPD2 were increased with IG alcohol feeding in mice (Fig 6C). GPD2 in mice was seen primarily as a monomer, unlike in rats where it is was mainly observed as a dimer. These findings suggest that up-regulation of GPD2 in liver mitochondria may be a key adaptation to chronic alcohol feeding in both mice and rats.

Effect of alcohol feeding on NAD^+-NADH levels and redox status in isolated liver mitochondria Because NAD^+ is the rate limiting substrate in alcohol metabolism, NAD^+-NADH levels and redox status were examined in isolated liver mitochondria following oral and IG alcohol feeding to rats. In mice, we previously observed that both oral and IG alcohol feeding increased total NAD^+-NADH levels in liver mitochondria [7]. In rats, a similar pattern was observed, as both oral and IG alcohol feeding significantly increased the total NAD^+-NADH levels in liver mitochondria (oral - 27%, IG - 48%; Fig 7). Since alcohol metabolism depends on NADH shuttling to regenerate NAD^+, an increase in the NAD^+-NADH pool in liver mitochondria could potentially enhance alcohol metabolism through increased NADH cycling [7]. The major difference observed between oral and IG alcohol fed rats was the NADH/NAD^+ ratio (Fig 7). In oral alcohol fed rats, mitochondria had a significantly greater NADH/NAD^+ ratio, suggesting a possible buildup of NADH from increased ADH activity and inhibition of respiration through complex I. IG alcohol feeding, which is associated with greater alcohol intake but enhanced respiration through complex I, resulted in the NADH/NAD^+ ratio being similar to control.

Effect of alcohol feeding on NADP+-NADPH levels and redox status in isolated liver mitochondria

NADPH plays an important role in biosynthesis, redox status, and alcohol metabolism (cytochrome P450-dependent metabolism) in cells and mitochondria [30]. Oral alcohol feeding was found to significantly increase $NADP^+$, NADPH, and total $NADP^+$-NADPH levels in liver mitochondria, while IG alcohol feeding was found to significantly increase NADPH, and total $NADP^+$-NADPH levels in liver mitochondria (Fig 8). Alcohol feeding did not significantly affect the NADPH/$NADP^+$ ratio in mitochondria, which generally favors the reduced form. An enhancement of mitochondrial NADPH by alcohol feeding may be an important adaptation to oxidative stress and redox alterations caused by alcohol.

Effect of oral and IG alcohol feeding on oxidative damage and N-acetylation in rat liver

We next examined the differences in post-translational protein modifications that oral and IG alcohol feeding caused in rats. Previously, we demonstrated that N-acetylation of proteins (lysine residues) in isolated mitochondria was increased with both oral and IG alcohol feeding to mice [7]. In rats, both oral and IG alcohol feeding significantly increased N-acetylation in mitochondrial proteins compared to their respective controls (Fig 9A, C). N-acetylation induced by alcohol feeding occurred primarily in mitochondrial proteins (25-150 kD range), suggesting preferential targeting. N-acetylation appears to increase equally with both oral and IG alcohol feeding in rats, in contrast to mice where N-acetylation was greater with IG feeding. Oral fed control rats had greater N-acetylation in the liver than IG controls, suggesting that feeding models influence N-acetylation irrespective of alcohol. The levels of N-acetylation in IG controls were similar to untreated rats (ad-libitum; data not shown), suggesting dietary changes associated with oral feeding enhances N-acetylation in liver mitochondria.

We also measured 4-hydroxynonenal (HNE)-protein adducts in the liver to assess oxidative stress induced by alcohol. HNE-protein adducts increase with lipid peroxidation, as HNE is formed by the breakdown of lipid peroxides. Only one consistent HNE-protein adduct (~

250 kD) was observed in the liver, and only in the cytoplasmic fraction (Fig 9A-B). IG alcohol feeding but not oral alcohol feeding significantly increased formation of this HNE-adduct, supporting the idea that increased alcohol dosing enhances oxidative stress in the liver.

Discussion

Previous studies that have examined the effect of alcohol feeding on mitochondrial changes in the liver have mainly utilized the oral alcohol model in rats [15, 16]. Oral alcohol feeding to rats had been shown to cause significant declines in mitochondrial state III respiration, decreased expression of respiratory complex proteins, and declines in RCR, all of which were also observed in this study. These findings, along with other studies demonstrating other types of mitochondrial damage (i.e. mtDNA oxidation, post-translational modification, oxidative stress) have led to the widely accepted hypothesis that “mitochondrial dysfunction” is central in alcoholic liver disease [12, 13]. Our previous findings that alcohol feeding in mice causes mitochondrial remodeling and enhances mitochondrial respiration in liver mitochondria seem to contradict the mitochondrial dysfunction hypothesis based primarily on the oral alcohol rat model [7]. In this study, our comparison of oral versus IG feeding models in rats demonstrated that no relationship between mitochondrial dysfunction and alcohol dosing occurs. IG alcohol feeding to rats did cause greater oxidative stress (HNE-protein adducts), but also caused less mitochondrial dysfunction (i.e. succinate driven-respiration), and greater mitochondrial remodeling (i.e. increased acetaldehyde-, octonoate-, GP3-driven respiration). Therefore, we believe that the “mitochondrial dysfunction” paradigm is an incomplete description of mitochondrial alterations that occur in the liver with alcohol feeding. While alcohol feeding causes some mitochondrial dysfunction and injury (i.e. succinate-driven respiration), our work suggests that the major consequence is mitochondrial remodeling in the liver as an adaptation to the stress induced by alcohol intake.

Effect of alcohol feeding on mitochondrial respiration - It is clear that feeding alcohol orally to rats causes a decline in many important bioenergetic parameters, including a decline in glutamate/malate- and succinate-driven respiration in liver mitochondria. These declines in mitochondrial respiration following oral alcohol feeding have generally been attributed to decreased expression of complexes I, III, IV, and V in liver mitochondria. However, mitochondrial respiration and levels of respiratory complex proteins do not seem to necessarily correlate in rats, as there is no decline in expression of complex II even though succinate-driven respiration is the most inhibited with alcohol feeding. Although declines in respiratory complex proteins and mitochondrial respiration can imply dysfunction, these changes may also be indicative of mitochondrial remodeling. Alcohol feeding is associated with excess or sufficient energy intake, and therefore, it is possible that the liver may be downregulating components of the electron transport chain as a response to excess energy levels from alcohol intake. It may also be that complex I respiration may be downregulated, so that other respiratory pathways including GPD2 and beta-oxidation are enhanced. Since NADH generated in the cytoplasm by ADH cannot cross the mitochondrial inner membrane, GPD2 is an important pathway in shuttling electrons from cytoplasmic NADH into mitochondria to regenerate NAD^+ for alcohol metabolism [30]. The enhanced G3P- and

octanoate-driven respiration may be particularly useful adaptations to alcohol feeding, since enhanced GPD2 may increase alcohol metabolism by ADH and enhanced beta-oxidation may help reduce fatty liver. Enhancement of octanoate-driven and G3P-driven respiration was also observed in the liver of mice fed alcohol intragastrically, suggesting that upregulation of these pathways are major adaptations to alcohol. Taken together, our work suggests that a significant number of mitochondrial alterations following alcohol feeding were due to mitochondrial remodeling, rather mitochondrial dysfunction.

One mitochondrial alteration in liver mitochondria that strongly supports mitochondrial dysfunction due to alcohol feeding was the decline in succinate-driven respiration. Both oral and IG alcohol feeding significantly decreased succinate-driven respiration, even though levels of complex II were unchanged (oral) or significantly increased (IG). Increased levels of complex II following IG alcohol feeding suggests that increased synthesis of complex II protein levels was compensating for an inhibition of complex II. Complex II may be inhibited by post-translational modifications to mitochondrial proteins, such as nitrosylation and N-acetylation, that have been shown to increase with alcohol feeding [31-33]. Our work shows that N-acetylation is increased with both oral and IG alcohol feeding, suggesting this protein post-translational modification may play a role in the decline in complex II activities that occurs with alcohol feeding. Interestingly, we observed that N-acetylation occurred equally with oral and IG alcohol feeding, which requires further investigation. The targeting of N-acetylation to mitochondrial proteins and its mechanism of induction by alcohol feeding requires further investigation. Complex II has also been shown to be inhibited by oxidative stress through an oxaloacetate dependent pathway [34]. Further research is needed to understand the mechanism by which succinate-driven respiration is inhibited by oral and IG alcohol feeding in rats. In mice, succinate-driven respiration is enhanced with both oral and IG alcohol feeding, suggesting major species differences exist.

Effect of alcohol feeding on acetaldehyde-driven respiration - An important observation that led to the mitochondrial dysfunction hypothesis has been the observed decline in acetaldehyde-driven respiration in liver mitochondria following oral alcohol feedings to rats. Indeed, it is puzzling that metabolism of a key toxicity intermediate of alcohol metabolism would decline with alcohol feeding. However, we observed that the higher alcohol delivery associated with IG alcohol feeding enhanced acetaldehyde-driven respiration in liver mitochondria. This implies that at a higher alcohol dose, which generates higher levels of acetaldehyde, liver mitochondria adapt and metabolize acetaldehyde at a greater rate. Acetaldehyde respiration and metabolism measurements are usually performed using high micromolar concentrations of acetaldehyde. It may be that oral alcohol feeding does not generate high enough levels of acetaldehyde in the liver to sufficiently decrease its metabolism even when complex I expression is suppressed. It was surprising that IG alcohol feeding enhanced acetaldehyde-driven respiration considering that protein levels of complexes I and III declined in liver mitochondria. However, mitochondria in rats have been shown to have excess respiratory complex proteins [35] and mitochondria tend to exhibit a “mitochondrial threshold effect” [36]. It has been estimated that up to 70% of complexes III and IV may be reduced before mitochondrial respiration is inhibited in rat brain and muscle mitochondria [35]. Thus, it is possible that acetaldehyde driven-respiration is enhanced even when expression of respiratory complexes are suppressed due to increased substrate

transport into mitochondria, changes in Fe-S cluster levels in the respiratory complexes, formation of supercomplexes, increased ALDH2 activity, or increased levels of pyridine nucleotides. In addition, although our immunoblotting data correlates well with previously published BN-PAGE data, by measuring only key subunits of the respiratory complexes, we may be missing protein alterations in the complexes that may help enhance acetaldehyde-driven respiration. Overall, the excess respiratory proteins observed in rat mitochondria may help explain why changes in respiratory complex protein levels often did not correlate with changes in mitochondrial respiration following alcohol feeding to rats. In mice, there are much stronger correlation between changes in mitochondrial respiration and changes in respiratory complex levels in the liver following alcohol feeding. Further research is needed to characterize the mitochondrial threshold effect in liver mitochondria in both mice and rats, and how it may be affected by alcohol feeding.

Decline in RCR in liver mitochondria caused by alcohol feeding - In all respiratory substrates examined there was a trend for a decline in RCR, with the decline being statistically significant with succinate in IG alcohol fed rats, and glutamate/malate for oral alcohol fed rats. A decline in RCR suggests that mitochondria are more uncoupled and have reduced ATP production capacity. Since mitochondria can become uncoupled following mitochondrial injury or damage, it is often a sign of mitochondrial dysfunction. However, uncoupling of mitochondria also has physiological functions. Mitochondrial uncoupling can be induced and regulated by the family of uncoupling proteins (UCP) [37]. The physiological functions of mitochondrial uncoupling include generation of heat and decreased mitochondrial ROS generation due to the shorter half-life of the ubisemiquinone radical responsible for ROS generation [38, 39]. Since alcohol feeding causes excess energy and leads to increased fatty acid formation, ATP levels are not limiting in the liver. Thus, under conditions of excess energy, uncoupled mitochondria may be a form of adaptation to quickly regenerate NAD^+ needed for alcohol metabolism, without generating excess ATP and generating less ROS. Further research is needed to determine if declines in mitochondrial RCR caused by alcohol are due to mitochondrial damage, or an adaptation possibly involving UCP.

Increased levels of pyridine nucleotides in liver mitochondria - Both oral and IG alcohol feeding to rats increased the NAD^+-NADH pool and $NADP^+$-NADPH pool in liver mitochondria. Since alcohol and acetaldehyde metabolism depends on NADH shuttling to regenerate NAD^+, an increase in the NAD^+-NADH pool in liver mitochondria could potentially contribute to enhanced NADH cycling and alcohol metabolism. Increased levels of mitochondrial NAD^+-NADH could also potentially be contributing to enhanced acetaldehyde-driven respiration observed with IG alcohol fed rats despite decreased expression of complexes I and III in liver mitochondria. Another interesting observation seen in this work was that oral alcohol feeding increased the NADH/NAD^+ ratio in liver mitochondria, suggesting a buildup of NADH from alcohol metabolism. It is possible that the decreased respiration through complex I may be contributing to a buildup of NADH in oral alcohol fed rats. With oral alcohol feeding, there seems to be an inability to achieve the normal NADH/NAD^+ ratio, suggesting mitochondrial stress. However, with IG feeding, liver mitochondria seem to adapt, with complex I respiration being greater than control

(acetaldehyde-driven respiration), causing the NADH/NAD^+ ratio to increase only slightly above basal levels.

NADPH plays an important role in biosynthesis and in maintaining the redox status in cells and mitochondria [30, 40]. Consequently, it is not surprising that both oral and IG alcohol feeding enhanced NADPH and total $NADP^+$-NADPH levels in liver mitochondria. Alcohol feeding is known to generate oxidative stress and alter the redox status of proteins in mitochondria [41], and increased NADPH levels are likely an adaptive response. NADPH is also the key substrate for cytochrome P450s, such as CYP2E1, that help metabolize alcohol and are upregulated with alcohol feeding. The effect of alcohol intake on pathways, such as mitochondrial nicotinamide mononucleotide adenylyltransferase (NMNAT-3) and NAD^+ kinase (NADK) that synthesizes NADPH needs to be further explored [30]. Overall, the increased levels of pyridine nucleotides in liver mitochondria may be an important adaptive mechanism to help with alcohol metabolism and alcohol detoxification.

Model of mitochondrial remodeling

Alcohol feeding clearly causes some mitochondrial dysfunction in rats, such as a decline in succinate-driven respiration. Alcohol feeding has also been shown to increase mitophagy in the liver to remove damaged mitochondria [42]. While mitochondrial dysfunction is important in alcoholic liver disease, it represents an incomplete picture of mitochondrial dynamics that occur in the liver following alcohol feeding. Our data suggests that mitochondria also adapt and undergo dynamic alterations with chronic alcohol feeding. In mice, we see dramatic mitochondrial remodeling that enhances mitochondrial respiration over 2-fold. In rats, the mitochondrial remodeling is not as dramatic, but we do observe increases in pyridine nucleotide levels and increased G3P-, octanoate-, and acetaldehyde-driven respiration (IG only). Thus, we are proposing a model in which alcohol feeding generates stress and mitochondrial injury. Following this stress and injury, mitochondria remodel to adapt to the stress of alcohol feeding.

Mitochondrial remodeling in the liver varies depending on the dose of alcohol intake (the greater the alcohol content the greater the remodeling) and between species (rats and mice). Mitochondrial remodeling is therefore likely to be highly variable in a heterozygous human population, depending on the genetic composition of patients and doses of alcohol consumed. It may be that some mitochondrial alterations have greater pathological consequences that promote liver injury through mechanisms such as increased ROS generation. G3P-driven respiration has been shown to have higher ROS generation than other respiratory pathways [43]. In support of this notion, ROS generation appears to increase with IG alcohol feeding, as evidenced by the fact that HNE-protein adduct formation in the liver increased with IG feeding. However, since alcohol metabolism by cytochrome P450 and other pathways can also increase ROS generation, further work is needed to determine the contribution that mitochondrial remodeling is making to the increased ROS generation observed with IG alcohol feeding. Mitochondrial remodeling could also potentially be involved in the increased sensitivity of liver mitochondria to mitochondrial permeability transition (MPT) that is observed with oral alcohol feeding [44].

Therefore, mitochondrial remodeling may help the liver adapt to alcohol in the short-term, but long-term may contribute to liver injury, which needs to be further explored.

In many ways, chronic alcohol feeding is analogous to exercising the liver. Exercise in muscle is associated with significant mitochondrial remodeling as an adaptation [2]. Exercise is also a stress in muscles and is associated with increased ROS [45]. Alcohol can be seen as exercising the liver by stressing the metabolic pathways of the liver and promoting ROS generation to trigger mitochondrial remodeling. It has been well established that the liver adapts to alcohol feeding, and rapidly develops an enhanced capacity to metabolize alcohol [46]. Mitochondrial remodeling may play an important role in enhanced alcohol metabolism and other adaptations that the liver develops with alcohol intake.

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Highlights

- Alcohol feeding to rats causes some mitochondrial dysfunction (*i.e.* inhibition of succinate-driven respiration) in the liver
- Alcohol feeding predominately causes mitochondrial remodeling (*i.e.* increased G3P-driven respiration, increased pyridine nucleotide levels, etc).
- Greater alcohol dosing by intragastric feeding increases mitochondrial remodeling, but not mitochondrial dysfunction
- Mitochondrial remodeling may play an important role in adaptation to stress induced by alcohol intake in the liver.

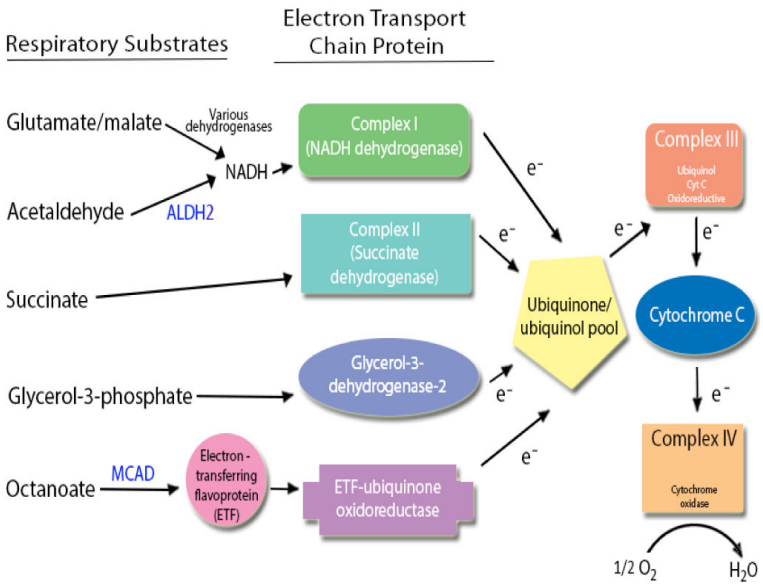

Figure 1. Respiratory substrates and proteins in the electron transport chain that feed electrons into ubiquinone

Many respiratory substrates can shuttle electrons into the four key proteins in the mitochondrial inner membrane that subsequently feed electrons into ubiquinone. Glutamate/malate treatment, through the action of various dehydrogenases such as glutamate and malate dehydrogenase, generates NADH that feeds into complex I. Acetaldehyde treatment also generate NADH, through the action of ALDH2, that also feeds into complex I. Succinate feeds into complex II (succinate dehydrogenase). The glycerol phosphate shuttle transfers electrons from NADH generated in the cytoplasm into the electron transport chain. In the glycerol phosphate shuttle, cytoplasmic glycerol phosphate dehydrogenase-1 (GPD1) transfers electrons from NADH to dihydroxyacetone phosphate to form glycerol-3-phosphate (G3P). Thus, G3P then feeds electrons into the respiratory chain through the action of mitochondrial glycerol phosphate dehydrogenase-2 (GPD2) located in the inner membrane. Thus G3P is a respiratory substrate for GPD2. Beta-oxidation of fatty acids generates $FADH_2$ through the action of various acyl-coenzyme A dehydrogenases, such as medium-chain acyl-coenzyme A dehydrogenase (MCAD). Electrons from $FADH_2$ are shuttled into the respiratory chain through electron-transferring flavoprotein (EFT) and EFT-ubiquinone oxidoreductase. Octanoate-driven respiration, therefore, depends on many proteins including MCAD.

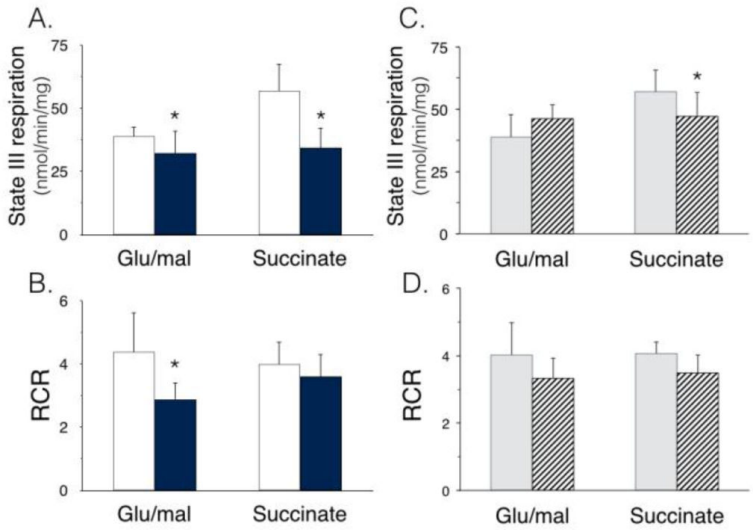

Figure 2. Effect of oral and IG alcohol feeding on respiration through complex I and II in isolated rat liver mitochondria

A) State III respiration in oral alcohol fed rats. White bars = control; Dark bars = oral alcohol treatment. B) Respiratory control ratio (RCR) in oral alcohol fed rats. C) State III respiration in IG alcohol fed rats. Gray bars = control; Stripped bars = IG alcohol treatment. D) RCR in IG alcohol fed rats. State III respiration was measured using either complex I substrates (glutamate/malate, 7.5 mM) or complex II substrate (succinate, 7.5) plus ADP (250 uM) with an oxygen electrode. RCR is defined as state III respiration/state IV respiration ratio. Following 6 weeks of oral alcohol feeding or 6 weeks of IG alcohol feeding, liver mitochondria were isolated using differential centrifugation as described in the materials and methods section. N = 6-8 rats per group. Results are mean ± SD; * $p < 0.05$ versus control. Controls were fed an isocaloric substitution of dextrin for alcohol.

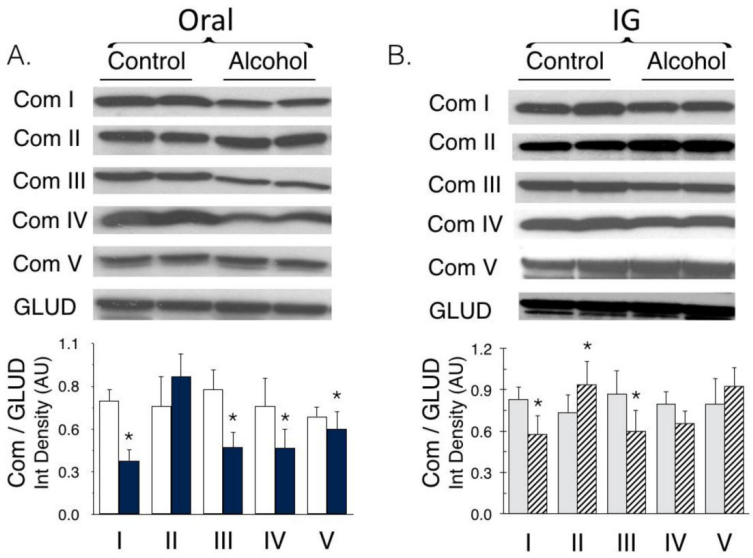

Figure 3. Alcohol feeding alters protein levels of respiratory complexes in the electron transport chain of rat liver mitochondria

A) Oral alcohol fed rats. White bars = control; Dark bars = oral alcohol treatment. B) IG alcohol fed rats. Gray bars = control; Stripped bars = IG alcohol treatment. Following 6 weeks of oral alcohol feeding or 6 weeks of IG alcohol feeding, mitochondria were isolated using differential centrifugation, and protein levels were assessed by immunoblotting. Densitometry was performed using Image J. Glutamate dehydrogenase (GLUD) was used as the loading control after it was observed not to change with alcohol feeding. Com = complex. N = 6-8 rats per group. Results are mean ± SD; * $p < 0.05$ versus control.

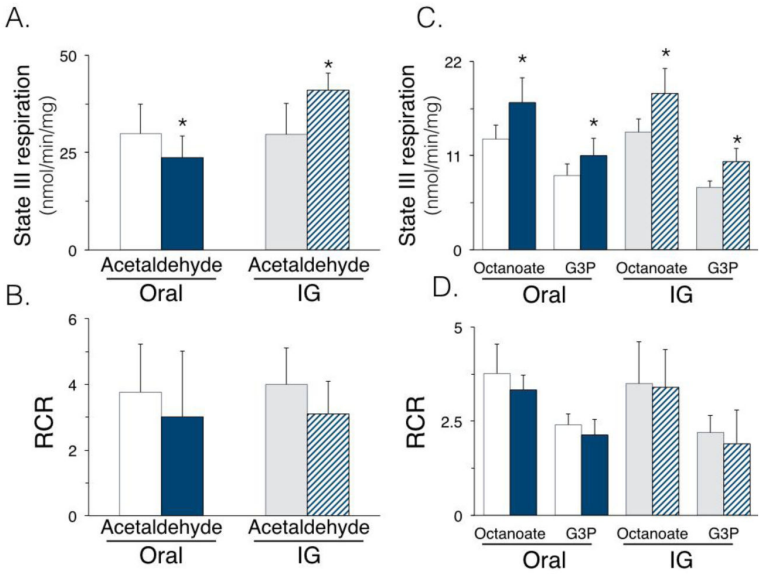

Figure 4. Effect of oral and IG alcohol feeding on acetaldehyde-, G3P-, and octanoate-driven respiration in isolated rat liver mitochondria

A) Acetaldehyde-driven state III respiration. White bars = oral control, Dark bars = oral alcohol treatment; Gray bars = IG control; Striped bars = IG alcohol treatment. B) RCR using acetaldehyde as substrate. C) G3P-and octanoate-driven state III respiration. D) RCR using G3P and octanoate as substrates. State III respiration was measured using either acetaldehyde (180 uM), G3P (2.5 mM), or octanoate (200 uM) plus ADP (250 uM) with an oxygen electrode. Following 6 weeks of oral alcohol feeding or 6 weeks of IG alcohol feeding, liver mitochondria were isolated using differential centrifugation. N = 6-7 rats per group. Results are mean ± SD; * $p < 0.05$ versus control.

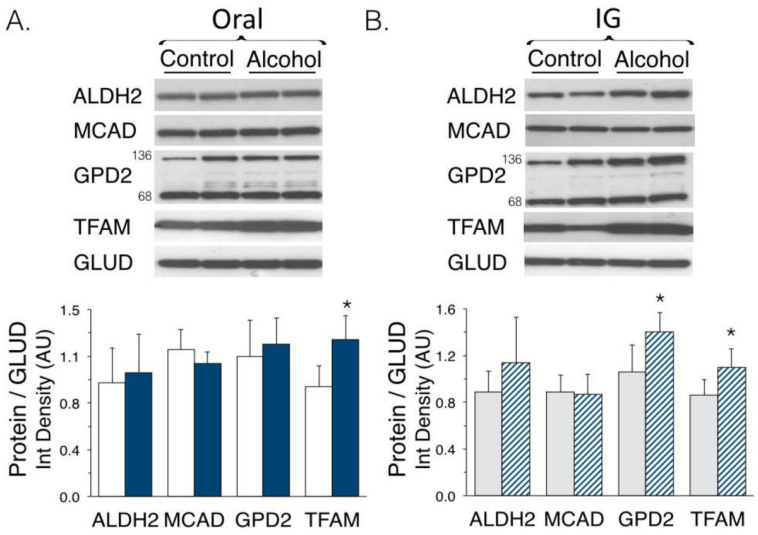

Figure 5. Effect of alcohol feeding on expression of mitochondrial proteins in the liver of rats

A) Oral alcohol fed rats. White bars = control; Dark bars = oral alcohol. B) IG alcohol fed rats. Gray bars = control; Striped bars = IG alcohol. Following 6 weeks of oral alcohol feeding or 6 weeks of IG alcohol feeding, mitochondria isolated using differential centrifugation, and mitochondrial protein levels were assessed by immunoblotting. Densitometry was performed using Image J. Glutamate dehydrogenase (GLUD) was used as the loading control. ALDH2 - aldehyde dehydrogenase 2; MCAD - medium-chain acyl-coenzyme A dehydrogenase; GPD2 - mitochondrial glycerol phosphate dehydrogenase-2, TFAM - mitochondrial transcription factor A. N = 6 rats per group. Results are mean ± SD; * $p < 0.05$ versus control.

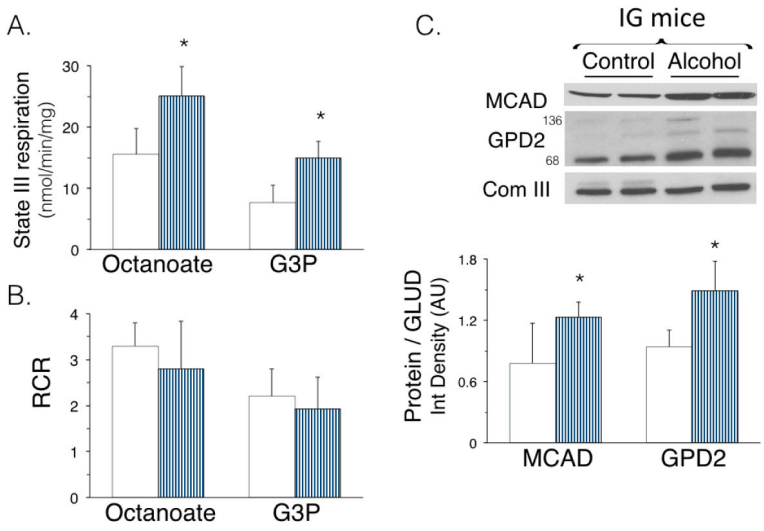

Figure 6. Enhanced G3P-, and octanoate-driven respiration is also observed in murine liver mitochondria following IG alcohol feeding

A) G3P and octanoate-driven state III respiration in isolated liver mitochondria from mice. Empty bars = control mice; Vertical striped = IG mice B) RCR using G3P and octanoate as substrates. C) Effect of IG alcohol feeding on MCAD and GPD2 expression in isolated mice liver mitochondria. State III respiration was measured using either G3P (2.5 mM), or octanoate (200 uM) plus ADP (250 uM) with an oxygen electrode. Following 5 weeks of IG alcohol feeding, liver mitochondria were isolated using differential centrifugation. Complex III (com III) was used as the loading control, since we previously observed that it does not change in mice with alcohol feeding. N = 7-8 mice per group. Results are mean ± SD; * $p < 0.05$ versus control.

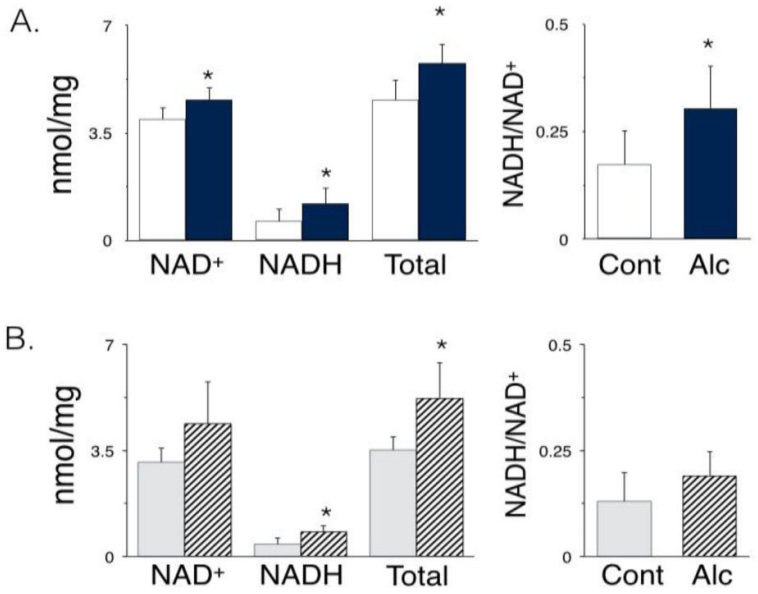

Figure 7. Effects of oral and IG alcohol feeding on NAD^+-NADH levels and redox status in liver mitochondria

A) Oral alcohol fed rats. White bars = control; Dark bars = oral alcohol treatment. B) IG alcohol fed rats. Gray bars = control; Striped = IG alcohol treatment. Following 6 weeks of oral alcohol feeding or 6 weeks of IG alcohol feeding, mitochondria isolated using differential centrifugation. NAD^+ and NADH levels were measured by HPLC with the fluorescence detector. N = 5-6 rats per group. Results are mean ± SD; * $p < 0.05$ versus control.

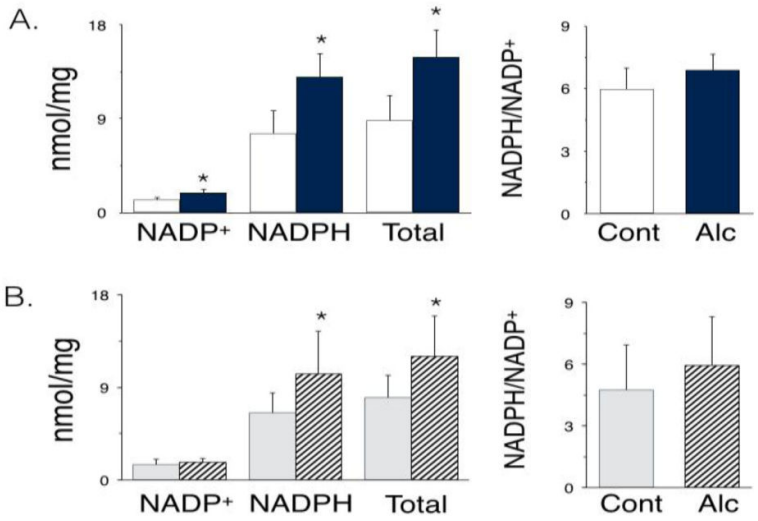

Figure 8. Alcohol feeding to rats increases mitochondrial $NADP^+$-NADPH levels in the liver

A) Oral alcohol fed rats. Gray bars = control; Dark bars = oral alcohol treatment. B) IG alcohol fed rats. Empty bars = control; Dark patterned bars = IG alcohol treatment. Following 6 weeks of oral alcohol feeding or 6 weeks of IG alcohol feeding, mitochondria were isolated using differential centrifugation. $NADP^+$ and NADPH levels were measured by HPLC with the fluorescence detector. N = 5-7 rats per group. Results are mean ± SD; * $p < 0.05$ versus control.

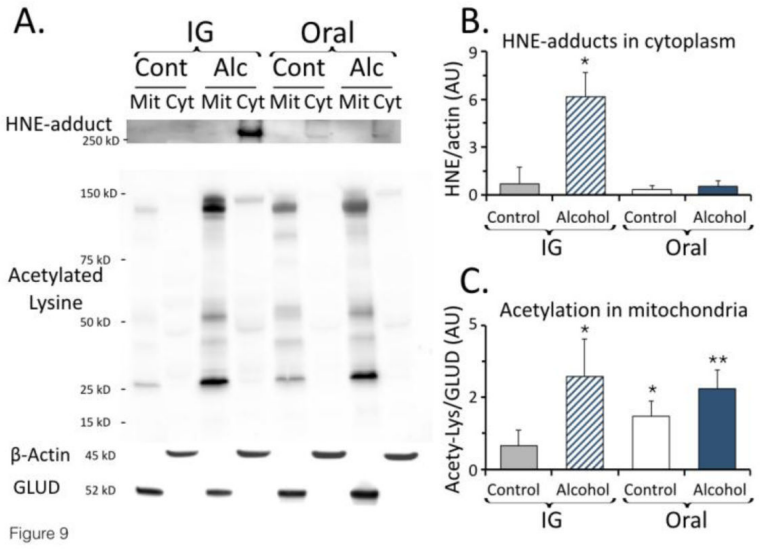

Figure 9. Effect of oral and IG alcohol feeding on oxidative damage and *N*-acetylation in cytoplasmic and mitochondrial fractions of rat liver

A) Following 6 weeks of oral alcohol feeding or 6 weeks of IG alcohol feeding, mitochondrial and cytoplasmic (soluble fraction minus mitochondria) fractions were isolated using differential centrifugation. Levels of HNE-protein protein adducts and *N*-acetylation (lysine) were assessed by immunoblotting. Densitometry was performed using Image J. Glutamate dehydrogenase (GLUD) was used as the mitochondrial loading control. Actin was used as the cytoplasmic loading control. Mit = mitochondrial fraction. Cyt = cytoplasmic fraction. Results are mean ± SD; * $p < 0.05$ versus IG control. ** $p < 0.05$ versus IG control. N = 4-7 rats per group.

Table 1

Effect of oral and intragastric alcohol feeding on serum ALT levels and hepatomegaly in rats

	ALT	Body Weight	Liver Weight	Liver/Body Weight (%)
Control (oral)	28.9 + 10.2	417.7 + 15.0	13.1 + 1.29	3.13 + 0.29
Oral Alcohol	47.6 + 5.4 *	374.1 + 36.1 *	13.9 + 0.9 *	3.72 + 0.32 *
Control (IG)	27.5 + 6.46	458.9 + 27.4	15.4 + 1.3	3.36 + 0.25
Intragastric Alcohol	65.7 + 21.7 *,^	416.4 + 25.6 *,^	18.0 + 1.8 *,^	4.33 + 0.38 *,^

N = 6-8 mice per group. Results are mean + SD;

* $p < 0.05$ versus pair fed controls,

^ $p < 0.05$ versus oral alcohol fed rats