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Ethanol feeding enhances age-related deterioration of the rat hepatic mitochondrion

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Abstract

Chronic ethanol feeding damages the hepatic mitochondrion by increasing mitochondrial DNA (mtDNA) oxidation, lowering mtDNA yields and impairing mitochondrial respiration. These effects are also seen during aging. By employing a 21-day chronic feeding regimen, we investigated the effects of ethanol consumption on mtDNA content and mitochondrial respiration in 2-, 12-, and 24-mo-old male rats. Aging resulted in decreased mtDNA content, increased mtDNA damage (as indicated by inhibition of *Taq* polymerase progression), and a decline in state 3 respiration; effects that were further exacerbated by ethanol feeding. Additionally, ethanol consumption caused an increase in the levels of citrate synthase while not impacting mitochondrial protein content. In conclusion, ethanol and aging combine to cause deterioration in the structural and functional integrity of the hepatic mitochondrion. The additive effects of aging and ethanol feeding may have serious consequences for hepatic energy metabolism in aged animals, and their detrimental combination may serve as one of the molecular mechanisms underlying the progression of alcoholic liver disease.

Keywords

mitochondrial DNA; respiration; polymerase-blocking lesions; citrate synthase

CHRONIC ETHANOL FEEDING results in a number of detrimental alterations to the structural and functional integrity of the hepatic mitochondrion, e.g., altered morphology (17,20), impaired mitochondrial protein synthesis (8), decreased activity/levels of electron transport chain components (14,15), and potentiation of mitochondrial permeability transition (47). Additionally, ethanol feeding, both chronic and acute, leads to oxidative damage to, and decreased yields of, hepatic mitochondrial DNA (mtDNA) (9,10,38,39). Many of these ethanol-elicited deleterious effects on energy metabolism can also be seen during aging, e.g., altered mitochondrial morphology (52), impaired electron transport chain activity (43), increased susceptibility to mitochondrial permeability transition (22,40), and impaired structural integrity of hepatic mtDNA (6,32). These observations lend themselves to the intriguing possibility that a similar molecular mechanism(s) may underlie both processes. Thus far, no studies have specifically investigated the potential interaction of ethanol treatment and the aging process and how such an interaction may compromise mitochondrial function. To that end, we present the following data showing how aging results in a gradual deterioration in the structural and functional integrity of the hepatic mitochondrion, and we demonstrate that ethanol feeding increases the rate of this deterioration.

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EXPERIMENTAL PROCEDURES

Reagents and chemicals

All reagents were molecular biology grade and obtained from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Animal experimentation and feeding regimen

Male Fischer 344 × Brown Norway (F344BN) rats aged 2, 12, and 24 mo were obtained from the National Institute on Aging (Bethesda, MD) and maintained on a liquid diet (35) with ethanol constituting 36% of the calories for 21 days (48). This feeding regimen is referred to as the short-term chronic feeding regimen. In some experiments, male Sprague-Dawley rats aged 2 and 12 mo were fed ethanol for 12 and 2 mo, respectively, whereas in other experiments 2-mo-old male Sprague-Dawley animals were fed ethanol for 2 and 18 mo. These 12- and 18-mo ethanol feeding regimens are referred to as the long-term chronic feeding regimen. Control animals were pair fed the same diet but with maltose-dextrin isocalorically substituted for ethanol. Animals received humane care according to criteria outlined in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Liver histology

Tissue was fixed in formalin and embedded in paraffin. Sections (5–6 μ m thick) were cut and stained with hematoxylin and eosin.

Isolation of mtDNA and genomic DNA

Hepatic mitochondria were isolated by differential centrifugation (13) and incubated in the presence of DNase I (10). mtDNA was isolated using a Qiagen Plasmid Midi Kit (Qiagen; Valencia, CA) (10) and dissolved in either TrisEDTA [10 mmol/l Tris-HCl (pH 8.0) and 1 mmol/l EDTA] buffer or autoclaved water depending on the subsequent application. Total genomic DNA was extracted from liver samples using the QIAamp Tissue Kit (Qiagen) according to the supplier's protocol. All DNA samples were stored at -20°C until use.

Quantification of DNA and protein samples

DNA samples were quantified by monitoring 260-nm absorbance values or by the Picogreen assay (Molecular Probes; Eugene, OR). For long PCR analysis of mtDNA, samples were prequantified using the Picogreen assay and diluted to 10 ng/ μ l. Samples were then requantified to a final concentration of 1 ng/ μ l. Protein levels were determined using the Bio-Rad Detergent-Compatible Protein Assay Kit (Bio-Rad Laboratories; Hercules, CA) with BSA as the protein standard.

Long PCR

Isolated mtDNA was further cleaned using the Wizard DNA Clean-Up system (Promega; Madison, WI) and amplified (1 and 2 ng) between nucleotides 15123 and 14132 (primers: 5'-gtcttaacatgaatcggaggccaacc-3' and 5'-tgaatagggggtgagatttcggatg-3'). PCR conditions comprised sequentially 75°C for 2 min, 94°C for 1 min, 18 cycles of 94°C for 15 s, and 65°C for 13 min, followed by a 10-min extension at 72°C (51). Products were electrophoresed through a 0.8% agarose gel containing 50 μ g/ml ethidium bromide in Tris-borate-EDTA (0.1 mol/l Trizma base, 90 mmol/l boric acid, and 1 mmol/l disodium EDTA) buffer. Amplified bands were visualized by ultraviolet transillumination and quantified using Kodak image station 440CF (Eastman Kodak; Rochester, NY). Assuming that the polymerase-blocking lesions were distributed randomly, the average number of lesions per strand (λ) of mtDNA at dose D was calculated using an equation based on the “zero class” of a Poisson distribution:

$\lambda = -\ln (A_D/A_0)$, where A_D is the total number of lesions after treatment and A_0 is the number of lesions without treatment.

Determination of mitochondrial respiration rates

Mitochondria were resuspended (0.5 mg/mL) in respiration buffer (130 mmol/L potassium chloride, 2 mmol/L potassium phosphate, 3 mmol/L HEPES, 2 mmol/L $MgCl_2$, and 1 mmol/L EGTA; pH 7.2) in a thermally jacketed electrode chamber. Oxygen utilization was measured with a Clarke electrode in the presence of succinate (10 mmol/l) and amytal (1 mmol/l). Respiration-coupled ATP synthesis was initiated by the addition of ADP (0.6 mmol/l).

Measurement of citrate synthase activity

Either isolated mitochondria or whole liver homogenate was assayed for citrate synthase (CS) activity according to the laboratory protocol of Kuznetsov et al. (33).

Statistical analyses

Statistical analysis of data was performed using Prism 4 (GraphPad Software; San Diego, CA) and Excel (Microsoft; Redmond, WA).

RESULTS

Effects of aging and ethanol consumption on fat deposition in the liver

Earlier studies performed in our laboratory suggested that chronic ethanol feeding has a deleterious effect on the structural integrity of rat liver mtDNA. One especially relevant observation was that, whereas there were no changes in mitochondrial protein levels, ethanol ingestion was associated with an ever-decreasing yield of mtDNA (9,10). In addition, preliminary experiments revealed that decreased hepatic mtDNA yields incurred during 12-mo chronic ethanol feeding of 2-mo-old male rats could be mimicked by 2-mo chronic ethanol feeding of 12-mo-old animals (Fig. 1). To further investigate this phenomenon, male rats of 2 (young), 12 (middle aged) and 24 (old) mo of age were maintained on a short-term chronic ethanol-containing diet for 21 days. Young animals increased their rate of ethanol-containing diet consumption by 56% from 45 to 70 ml/day over the course of the study in contrast to old animals, which maintained a steady rate of consumption (85 ml/day). Animals were then killed, and the livers were excised and stained with hematoxylin and eosin. Figure 2 shows representative micrographs of rat livers isolated from old and young animals fed ethanol or control diets. In the case of young controls (data not shown), young ethanol-fed controls (Fig. 2A), and old controls (Fig. 2B), no fat deposition occurred, and the livers displayed normal architecture. In the case of old animals maintained on an ethanol-containing diet, however, significant fat deposition was seen (Fig. 2C).

Effects of ethanol and aging on liver weight, mitochondrial protein yields, and CS activities

Ethanol feeding resulted in a 34% increase in liver weight (Fig. 3B) and liver-to-body weight ratio (Fig. 3C) in 2-mo-old animals compared with their pair-fed controls. These effects were not seen in older animals. Total hepatic mitochondrial protein yields increased by ~70% in 24-mo-old animals compared with 2-mo-old irrespective of ethanol treatment (Fig. 4A). When normalized for liver size, mitochondrial protein yields remained constant at 25 mg mitochondrial protein/g liver in all age groups except for the 2-mo-old ethanol-fed group, which showed a 21% decrease (Fig. 4B) due to the larger liver size (Fig. 3B). As an alternative to mitochondrial protein measurements, the effects of aging and ethanol feeding on hepatic CS activities were investigated. This tricarboxylic acid pathway enzyme is a classic mitochondrial marker enzyme, and its activity is often used to determine changes in mitochondrial content. Figure 5 shows that 2- and 12-mo ethanol feeding increased CS activity in the whole liver by

25% and 42%, respectively, with control values being consistent with those reported in the literature (42). Further analysis revealed that this increase was due to elevated CS activities within the mitochondria rather than an increase in mitochondria themselves (2-mo controls: 0.146 ± 0.008 U/mg mitochondrial protein vs. 2-mo ethanol-fed rats: 0.173 ± 0.006 U/mg mitochondrial protein, $P < 0.05$, $n = 7$; 12-mo controls: 0.1375 ± 0.007 U/mg mitochondrial protein vs. 12-mo ethanol-fed rats: 0.167 ± 0.013 U/mg mitochondrial protein, $P < 0.05$, $n = 4$; 1 unit = 1 μ mol citrate produced/min). Aging resulted in an 18% decrease in hepatic CS activity when it was normalized for liver size and a 6% decrease when it was normalized for mitochondrial protein content.

Effects of ethanol and aging on hepatic mtDNA content

Figure 6 depicts hepatic mtDNA yields normalized for mitochondrial protein (A) and liver weight (B). Aging resulted in a 75% decrease in mtDNA content in ethanol-fed animals over a time period of 24 mo and a corresponding 60% decrease in paired controls. Twenty-one days of ethanol feeding resulted in decreased mtDNA yields of ~ 25 ng/mg mitochondrial protein irrespective of the age of the animals (Fig. 6A). When expressed as a percentage of the paired controls, ethanol feeding decreased mtDNA yields by 23%, 36%, and 50% in 2-, 12-, and 24-mo-old animals, respectively.

Two-way ANOVA analyses of the interaction between ethanol feeding and aging in the short-term chronic ethanol feeding regimen

Table 1 displays a statistical analysis of data for liver weights, mitochondrial protein content, and mtDNA yields. Aging caused significant changes to rat and liver weights and to yields of mitochondria and mtDNA, whereas ethanol feeding significantly altered liver weights and mtDNA yields. Additionally, an interaction was detected between aging and ethanol feeding with regard to liver weight.

Effects of ethanol and aging on mtDNA integrity

Earlier studies have shown that long-term chronic ethanol feeding results in decreased mtDNA yields and increased levels of mtDNA oxidative damage, as represented by elevated levels of 8-hydroxydeoxyguanosine (8-OHdG) formation (9). To investigate the structural integrity of hepatic mtDNA isolated from animals maintained on the short-term feeding regimen, long PCR was employed. Equal amounts of mtDNA from ethanol-fed animals and their paired controls were amplified between nucleotides 15123 (cytochrome *b*) and 14132 (cytochrome *b*), representing 94% of the rat mitochondrial genome. The idea was that successful amplification of the 15309-bp fragment would likely be impaired by oxidative damage and other bulky polymerase-blocking lesions. Figure 7A shows representative data from 12- and 24-mo-old animals subjected to the short-term chronic feeding protocol. In all cases, amounts of 1 and 2 ng of mtDNA template were used to confirm that amplification was proceeding in a linear manner and that accurate comparisons between animals could be made. Only PCRs in which the use of 1 ng of template resulted in 45–55% of reactions in which 2 ng was utilized were subjected to further analysis. Aging significantly decreased long PCR amplification of mtDNA, and ethanol feeding further enhanced this effect in 24-mo-old animals (Fig. 7B). Analysis of the data using the Poisson equation revealed an ethanol-elicited increase in the number of polymerase-blocking lesions per mitochondrial genome of 0.22 ± 0.21 ($n = 4$) in 12-mo-old animals and 0.33 ± 0.09 ($P < 0.01$, $n = 4$) in 24-mo-old animals compared with their paired controls. Further analyses revealed that 12 mo aging increased the number of polymerase-blocking lesions per mitochondrial genome by 0.72 ± 0.27 ($P < 0.05$, $n = 4$) in ethanol-fed animals and 0.61 ± 0.17 ($P < 0.05$, $n = 4$) in their paired controls. It was concluded that aging results in an elevation in the number of polymerase-blocking lesions and that chronic ethanol feeding specifically exacerbates their formation in old animals.

Effects of ethanol and aging on mitochondrial respiration

Mitochondrial respiration was measured in 2- and 24-mo-old animals, fed ethanol for 21 days, in the presence of succinate, which is metabolized by succinate dehydrogenase, part of the succinate-Q reductase complex (complex II) of the electron transport chain. Amytal was present in the respiration chamber to inhibit electron transport from NADH to coenzyme Q. Ethanol feeding significantly decreased the respiratory control ratio (RCR) in both 2- and 24-mo-old animals (Fig. 8). This decrease was brought about by a significant depression in state 3 respiration. No ethanol-related effects were detected in state 4 respiration. In contrast, long-term chronic ethanol feeding for 18 mo significantly depressed the RCR by decreasing state 3 respiration and increasing state 4 respiration. Two-month chronic ethanol feeding also depressed the RCR, but this was solely due to decreased state 3 respiration. Statistical analyses of these data using two-way ANOVA (Table 2) revealed that both aging and ethanol feeding significantly decreased the RCR in both the short- and long-term ethanol feeding models by depressing state 3 respiration.

DISCUSSION

The mitochondrial theory of aging states that “the continuous production of free radical species by the mitochondrial electron transport chain over the lifetime of an organism eventually results in elevated mtDNA damage and a decline in mitochondrial respiratory function” (24). This can lead to impaired energy metabolism and increased cell death, especially during conditions of enhanced stress. One such stress condition may be that of chronic ethanol consumption. Long-term chronic ethanol feeding to male rats results in a number of deleterious alterations to the structural and functional integrity of hepatic mitochondria (see the INTRODUCTION). As with aging, chronic ethanol feeding leads to impaired hepatic energy metabolism, a deleterious process believed to contribute significantly to the progression of alcoholic liver disease. The interaction of chronic ethanol feeding and senescence, two seemingly dissimilar insults, and the consequences of their superimposition on the integrity of the liver mitochondrion was the focus of this study.

To determine the consequences of any potential aging/ethanol feeding interactions, a short-term chronic feeding model (21 days) was employed in 2-, 12-, and 24-mo-old male rats. The development of this feeding regimen arose as a direct consequence of our earlier studies, when it was discovered that short-term chronic ethanol feeding of older animals depleted hepatic mtDNA levels to a similar extent as long-term chronic feeding of younger animals (Fig. 1). Histological examination of the livers revealed a marked increase in fat deposition specifically in the 24-mo-old animals. The fact that old animals fed a control diet did not develop fatty livers suggests that the aging process predisposes the liver to ethanol-induced fat deposition. Although fatty liver is generally believed to be a benign process, it can develop into steatohepatitis, followed by severe hepatocellular damage and cirrhosis; as such, its presence in old animals fed ethanol for only 21 days should be viewed as potentially damaging to the liver. Figure 3 and Table 1 show a clear interaction existing between ethanol feeding and the age of the animal with regard to hepatomegaly. Chronic ethanol consumption is associated with an increase in hepatocyte volume due to increased intracellular water retention (28), decreased protein secretion (5), impaired protein catabolism (18), and elevated fat deposition (45). Young animals fed ethanol for 21 days showed significantly increased liver weights (Fig. 3B) and, consequently, liver-to-body weight ratios (Fig. 3C), whereas no such changes were seen in older animals. These observations have previously been reported on by Britton et al. (7), but, to date, little is known about the underlying mechanism(s).

The relationship among mtDNA damage, mtDNA levels, and electron transport chain activity is a difficult one to dissect. Figure 8 shows the respiration rates, in the presence of succinate, for mitochondria isolated from young and old animals maintained on the short- and long-term

chronic ethanol feeding regimen. Both aging and ethanol feeding resulted in a significant decrease in the RCR (Fig. 8C). Aging alone caused a significant decrease in both state 3 and state 4 respiration (Fig. 8, A and B), in agreement with other studies (23,61). Ethanol administration further repressed state 3 respiration irrespective of the feeding regimen (Fig. 8A) and additionally caused an increase in state 4 respiration after long-term consumption (Fig. 8B>). With regard to the elevated state 4 respiration, the underlying mechanism is not clear. Ethanol has been shown to upregulate the expression of uncoupling protein 2 (49) and to cause a buildup of bile acids within the liver (2,31). These phenomena have both been reported to impact mitochondrial bioenergetics by increasing state 4 respiration (27,50). The impairment of state 3 mitochondrial respiration seen during both the aging process and ethanol administration is likely to be the result of a number of superimposing mechanisms, e.g., hypothyroidism (37), decreased cytochrome *c* oxidase activity (56), and hypoxia-induced increases in nitric oxide production (12,54,60). In the case of nitric oxide production, its elevation is also accompanied by increased oxidative stress and decreased mitochondrial reduced glutathione levels, two phenomena often detected during chronic ethanol feeding (19,21,25).

Previous results have shown that chronic ethanol feeding alters the structural integrity of hepatic mtDNA along with its content (9,10) but does not have any effect on mitochondrial protein levels; this study further reinforces those observations. Both aging and ethanol feeding resulted in decreased yields of mtDNA when it was expressed per gram of liver (Fig. 6B) or per milligram of mitochondrial protein (Fig. 6A), whereas no changes in total mitochondrial protein were observed (Fig. 4, A and B). These data, taken with the statistical analyses shown in Table 1, suggest that both aging and ethanol feeding act to decrease mtDNA yields and that no interaction between the insults exists. Either each process serves to decrease mtDNA yields by independent molecular mechanism(s) or they do so through related mechanisms that, when superimposed, become additive. As an alternative method for the normalization of mitochondrial content, CS activities were measured. This tricarboxylic acid cycle enzyme is commonly utilized as a marker for mitochondrial content. Surprisingly, ethanol was found to significantly increase hepatic CS activity (Fig. 5) irrespective of feeding duration, resulting in an even greater decrease in mtDNA yields when normalized for this enzyme. In contrast, aging resulted in decreased CS activity, a finding that is in agreement with recent literature demonstrating an age-related decline in a number of essential mitochondrial enzymes (44). The ethanol-elicited CS increase in the liver was found to be due to increased activity within the mitochondria and not an increase in the number of mitochondria. One possible explanation for this may be the effect of ethanol feeding on vitamin B₁₂ absorption through the gut. Ethanol has been shown to decrease the absorption of food-associated vitamin B₁₂ in humans (36), and vitamin B₁₂ depletion has been demonstrated to produce a two- to threefold increase in CS activity by increasing its rate of synthesis (42). An alternative possibility is that CS may be one of a battery of proteins whose transcription/translation is upregulated during chronic ethanol feeding.

To investigate whether the decreased mtDNA yields seen during aging and ethanol feeding were associated with damage to the mtDNA template, long PCR was employed to amplify the mitochondrial genome. In these experiments, the ease of amplification of the genome is an indicator of the structural integrity of mtDNA. Decreased amplification occurs due to the presence of damaging lesions that impede the progress of *Taq* polymerase, i.e., single-strand breaks and bulky adducts (29,41,62). Aging resulted in significant increases in the number of polymerase-blocking lesions per hepatic mitochondrial genome, with ethanol feeding exacerbating the damage (Fig. 7, A and B, and Table 2). This is indicative of both processes contributing to mtDNA damage. Aging is associated with the formation of a number of deleterious adducts of mtDNA including products of lipid peroxidation (53,59), oxidation (16,52), and alkylation (46), all adducts likely to impede the progress of *Taq* polymerase in the

polymerase inhibition assay (29). Additionally, evidence has shown that aging interferes with the import of two major mtDNA repair enzymes, i.e., 8-oxoguanine-DNA glycosylase and uracil-DNA glycosylase, into the mitochondrial matrix (58). Whether any other proteins required for mtDNA maintenance are excluded from the matrix during aging has yet to be determined. The combination of increased mtDNA damage and decreased accessibility of repair enzymes results in a gradual decline in the structural integrity of the mitochondrial genome over the course of an animals' lifespan. Ethanol feeding, on the other hand, exacerbates mitochondrial ROS production in hepatocytes (3) and decreases levels of glutathione (21,25) and glutathione peroxidase (4) in the hepatic mitochondrion. The combination of these ethanol-elicited phenomena, i.e., increased ROS production and decreased levels of a major enzyme involved in hydrogen peroxide removal, is likely to result in a significantly greater oxidative environment for mtDNA. Depending on the site of action, the results of ROS attack can be either oxidized bases or single-strand breaks. Earlier studies from our laboratory have shown increased levels of oxidized bases, most notably 8-OHdG, within the mitochondrial genome of rats chronically maintained on an ethanol-containing diet (9,10). It may be that the increased levels of 8-OHdG are sufficient to impede the polymerase, but this is unlikely as a number of polymerases, including Taq polymerase, have been shown to bypass 8-OHdG (55,63) and, in doing so, sometimes pair a dA residue opposite; thus 8-OHdG is more often associated with G→T transversions than replication blocks. A more plausible theory would be that the increase in ROS production seen in ethanol-fed animals (3) leads to an elevation in the levels of abasic sites, single-strand breaks, or bulky lesions. All of these mtDNA modifications would be expected to impede polymerase progression. When the results of our earlier studies are taken together with the data presented in this study (see RESULTS and Fig. 7), they provide strong evidence that ethanol consumption increases damage to mtDNA over and above that incurred during aging.

In conclusion, ethanol feeding and aging both act to decrease mitochondrial respiration, compromise the structural integrity of the mitochondrial genome, and reduce mtDNA yields, a schematic illustration of which is shown in Fig. 9. Although the underlying mechanism(s) of the two types of insult may differ, the fact that they act to perturb two of the most important components of the mitochondrion, i.e., the mitochondrial genome and the electron transport chain, may have serious ramifications for the liver, especially with regard to energy metabolism. Cellular maintenance requires a certain extent of energy metabolism, and a minimum threshold is likely to exist, below which viability cannot be maintained. The impact of ethanol feeding and the aging process on liver ATP levels is therefore an important question and is currently the subject of an ongoing study in our laboratory. Previous studies have demonstrated that both short-term ethanol feeding in young animals (57) and 24 mo of aging (1) significantly decrease rat liver ATP levels, but a paucity of data examining the combination of the two insults exists. If impaired mitochondrial function represents an underlying component of cellular aging, a concept proposed by a number of researchers, then ethanol feeding would be expected to contribute to the aging process. In this regard, ethanol may not be alone as a number of xenobiotics [e.g., steroids, analgesics (11,30)] and physiological conditions [e.g., lung cancer and pancreatitis (26,34)], have been shown to affect liver mitochondria in a similarly deleterious manner. Aging should therefore be viewed as an underlying disease state that can be impinged upon, both positively and negatively, by a multitude of external and internal cellular insults. The potential of aging to impact the ethanol-mediated impairment of energy metabolism is intriguing when related to the progression of alcoholic liver disease. The majority of human alcoholics only get irreparable liver damage during the later years of their life. It may be that the molecular mechanism(s) ultimately responsible for liver failure in these patients are as much related to underlying aging mechanism(s) as they are to alcohol consumption. Further studies are needed to establish the minimal threshold level of hepatic energy metabolism required for cell survival and the phenomena that impact it.

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Fig. 1.

Effects of short- and long-term chronic ethanol feeding on hepatic mitochondrial DNA (mtDNA) content of male Sprague-Dawley rats. Animals aged 2 and 12 mo were maintained on an ethanol-containing diet for 12 and 2 mo, respectively, as described in EXPERIMENTAL PROCEDURES. Results represent means $\pm$ SE of 4 animals aged 2 mo and 3 animals aged 12 mo. Statistical significance of the difference between ethanol-fed and pair-fed control animals (* $P < 0.05$ and ** $P < 0.01$) was calculated using the paired t -test.

Fig. 2. Effect of aging and short-term chronic ethanol consumption on fat deposition in livers of male Fischer 344 $\times$ Brown Norway (F344BN) rats. Animals were maintained on an ethanol-containing diet for 21 days, and livers were harvested and prepared for histological analyses as described in EXPERIMENTAL PROCEDURES. *A*: 2-mo-old animal fed ethanol for 21 days; *B*: 24-mo-old control animal; *C*: 24-mo-old animal fed ethanol for 21 days.

Fig. 3.

Effect of aging on ethanol-elicited changes in body and liver weights of male F344BN rats fed using the short-term chronic dietary regimen. *A*: animal weight; *B*: liver weight; *C*: liver-to-body weight ratio. Results represent means $\pm$ SE of 4 pairs of animals. Statistical significance of the difference between the ethanol-fed group and pair-fed control group (** $P < 0.01$) was calculated using the paired *t*-test.

Fig. 4. Effect of aging on ethanol-elicited changes in hepatic mitochondrial protein content in male F344BN rats fed using the short-term chronic dietary regimen. *A*: mitochondrial protein content per liver; *B*: mitochondrial protein content per unit of liver. Results represent means $\pm$ SE of 4 pairs of animals.

Fig. 5.

Effect of aging and ethanol feeding on hepatic citrate synthase (CS) levels (1 unit = 1 μmol citrate formed/min). Results represent means $\pm$ SE of 4 pairs of animals. Statistical significance of the difference between ethanol-fed and pair-fed control groups (** $P < 0.01$) was calculated using the paired t -test.

Fig. 6.

Effect of aging on ethanol-elicited alterations in hepatic mtDNA content of male F344BN rats fed using the short-term chronic dietary regimen. *A*: mtDNA content per milligram of mitochondrial protein; *B*: mtDNA content per unit of liver. Results represent means $\pm$ SE of 4 pair-fed animals. Statistical significance of the difference between ethanol-fed and paired control groups (* $P < 0.05$ and ** $P < 0.01$) was calculated using the paired *t*-test.

Fig. 7.

Effect of ethanol consumption on hepatic mtDNA integrity of 12- and 24-mo-old male F344BN rats fed using the short-term chronic dietary regimen. Long PCR was used to amplify mtDNA as described in EXPERIMENTAL PROCEDURES. *A*: agarose (0.75%) gel electrophoretic analysis of an amplified 15.3-kb mtDNA band from representative pair-fed groups of animals. C, control; E, ethanol-fed; 1–4, number of animal pair. *B*: graphical representation of amplified 15.3-kb mtDNA band intensities. Results represent means $\pm$ SE of 4 pair-fed animals. Statistical significance of the difference between the ethanol-fed group and paired control groups (* $P < 0.05$) was calculated using the paired *t*-test.

Fig. 8.

Effects of short- and long-term chronic ethanol feeding on succinate-dependent hepatic mitochondrial respiration in male rats. Mitochondria (0.5 mg/ml) isolated from F344BN rats aged 2 and 24 mo and Sprague-Dawley rats aged 2 and 18 mo were incubated in the presence of succinate (10 mM). *A*: state 3 respiration; *B*: state 4 respiration; *C*: respiratory control ratio. Results represent means $\pm$ SE of 4 pair-fed animals. Statistical significance of the difference between the ethanol-fed and paired-fed control groups (* $P < 0.05$ and ** $P < 0.01$) was calculated using the paired *t*-test.

Fig. 9. Relationship among aging, ethanol feeding, and mitochondrial deterioration. NO, nitric oxide; UCP2, uncoupling protein 2; Cyt oxidase, cytochrome *c* oxidase; T₃, triiodothyronine; LP, lipid peroxidation.

Table 1

Two-way ANOVA analyses of data obtained using the short-term chronic ethanol feeding model in F344BN rats

	Interaction	Treatment	Age
Rat weight	NS	NS	<0.0001
Liver weight	0.0197	0.0464	<0.0001
Liver-to-body weight ratio	<0.0001	<0.0001	<0.0001
Mitochondrial protein content per liver	NS	NS	0.0005
Mitochondrial protein content per gram of liver	NS	NS	NS
mtDNA per mitochondrial protein	NS	0.0043	<0.0001
mtDNA per gram of liver	NS	0.001	0.0001

Values shown are *P* values. F344BN rats, Fischer 344 × Brown Norway rats; mtDNA, mitochondrial DNA; NS, not significant.

Table 2

Two-way ANOVA analyses of respiration data

	Interaction	Treatment	Age	Days on Diet
Short-term chronic ethanol feeding of F344BN rats				
State 3	NS	0.0017	<0.0001	
State 4	NS	NS	0.02	
RCR	NS	0.0053	0.0036	
Long-term chronic ethanol feeding of Sprague-Dawley rats				
State 3	NS	0.0006		<0.0001
State 4	NS	NS		NS
RCR	NS	0.0221		0.0152

RCR, respiratory control ratio.