Vol. 38, No. 12
December 2014

ALCOHOLISM CLINICAL & EXPERIMENTAL RESEARCH

N-Acetylcysteine Inhibits the Up-Regulation of Mitochondrial Biogenesis Genes in Livers From Rats Fed Ethanol Chronically

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Background: Chronic ethanol (EtOH) administration to experimental animals induces hepatic oxidative stress and up-regulates mitochondrial biogenesis. The mechanisms by which chronic EtOH up-regulates mitochondrial biogenesis have not been fully explored. In this work, we hypothesized that oxidative stress is a factor that triggers mitochondrial biogenesis after chronic EtOH feeding. If our hypothesis is correct, co-administration of antioxidants should prevent up-regulation of mitochondrial biogenesis genes.

Methods: Rats were fed an EtOH-containing diet intragastrically by total enteral nutrition for 150 days, in the absence or presence of the antioxidant *N*-acetylcysteine (NAC) at 1.7 g/kg/d; control rats were administered isocaloric diets where carbohydrates substituted for EtOH calories.

Results: EtOH administration significantly increased hepatic oxidative stress, evidenced as decreased liver total glutathione and reduced glutathione/glutathione disulfide ratio. These effects were inhibited by co-administration of EtOH and NAC. Chronic EtOH increased the expression of mitochondrial biogenesis genes including peroxisome proliferator-activated receptor gamma-coactivator-1 alpha and mitochondrial transcription factor A, and mitochondrial DNA; co-administration of EtOH and NAC prevented these effects. Chronic EtOH administration was associated with decreased mitochondrial mass, inactivation and depletion of mitochondrial complex I and complex IV, and increased hepatic mitochondrial oxidative damage, effects that were not prevented by NAC.

Conclusions: These results suggest that oxidative stress caused by chronic EtOH triggered the up-regulation of mitochondrial biogenesis genes in rat liver, because an antioxidant such as NAC prevented both effects. Because NAC did not prevent liver mitochondrial oxidative damage, extra-mitochondrial effects of reactive oxygen species may regulate mitochondrial biogenesis. In spite of the induction of hepatic mitochondrial biogenesis genes by chronic EtOH, mitochondrial mass and function decreased probably in association with mitochondrial oxidative damage. These results also predict that the effectiveness of NAC as an antioxidant therapy for chronic alcoholism will be limited by its limited antioxidant effects in mitochondria, and its inhibitory effect on mitochondrial biogenesis.

Key Words: *N*-Acetylcysteine, Ethanol, Mitochondria, Liver, Oxidative Stress.

MITOCHONDRIAL BIOGENESIS IS defined as the growth and division of preexisting mitochondria (Ventura-Clapier et al., 2008). Mitochondrial biogenesis is triggered by environmental stresses that activate a transcriptional program that up-regulates the expression of mitochondrial biogenesis genes. These genes include coactivators such as peroxisome proliferator-activated receptor gamma-coactivator-1 alpha (PGC-1α; the master regulator of mitochondrial biogenesis) and DNA-binding transcription factors such as nuclear respiratory factor-1 (NRF1), nuclear respiratory factor-2 (NRF2), mitochondrial single-strand binding protein-1, and mitochondrial transcription factor A (TFAM; Hock and Kralli, 2009). Up-regulation of mitochondrial biogenesis genes has previously been observed after ethanol (EtOH) administration to experimental animals in: (i) mice administered EtOH intragastrically for 4 weeks (Han et al., 2012); (ii) mice administered EtOH by gastric intubation for 4 days (Demeilliers et al., 2002); (iii) mice fed EtOH orally for 7 weeks (Larosche et al., 2009); and (iv) rats fed EtOH intragastrically for 1 month (Oliva et al., 2008). Current experimental evidence suggests that increased mitochondrial biogenesis in EtOH exposure models may represent a metabolic adaptation in order to: (i) increase NADH oxidation by complex I to enhance EtOH and acetaldehyde metabolism (Han et al., 2012); (ii) increase the β-oxidation capacity of mitochondria in order to increase fatty acid utilization for energy (Han et al., 2012); (iii) promote the maintenance of intact mitochondrial DNA (mtDNA; Lee and Wei, 2005); and (iv) reduce the

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Received for publication January 21, 2014; accepted September 6, 2014.

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DOI: 10.1111/acer.12576

production of mitochondrial prooxidants by replacing dysfunctional mitochondria (Handy and Loscalzo, 2012). The mechanisms by which chronic EtOH up-regulates mitochondrial biogenesis genes have not been fully explored.

In several model systems, mitochondrial biogenesis is induced by conditions that increase the steady state levels of intracellular oxidants (i.e., oxidative stress). For example, mitochondrial biogenesis was induced in human lung fibroblasts exposed to hydrogen peroxide (Lee and Wei, 2005), in rat hepatoma cells exposed to tert-butyl hydroperoxide (Piantadosi and Suliman, 2006), in rat heart from animals exposed to lipopolysaccharide (Suliman et al., 2004), and in rat brain from animals exposed to cerebral ischemia (Chen et al., 2011), all conditions associated with oxidative stress. Signaling pathways that might mediate the induction of mitochondrial biogenesis following oxidative stress insults have been suggested to include p38 MAPK, PI3 kinase/AKT, protein kinase A, and calcium-dependent regulatory enzymes (Rasbach and Schnellmann, 2007a).

Prolonged EtOH administration induces hepatic oxidative stress, evidenced as increased steady state levels of reactive oxygen species (ROS) and reactive nitrogen species (Arteel, 2003). The mechanisms by which EtOH induces hepatic oxidative stress include an increase in the generation rate of prooxidants, and/or a decrease in antioxidant levels. EtOH induces an increase in the generation rate of hepatic prooxidants through enzymes at the level of Kupffer and inflammatory cells (including NAD[P]H oxidase [NOX enzymes] and inducible nitric oxide synthase; Loguercio and Federico, 2003), and at the level of hepatocytes (including cytochrome P450 2E1, NOXs, inducible nitric oxide synthase, and the mitochondrial electron transport chain; Cederbaum, 2009; De Mincis and Brenner, 2008). An EtOH-dependent decrease in antioxidant levels can be caused by nutritional deficiencies or alterations in glutathione homeostasis independent of nutritional status (Albano, 2006).

Considering this previous information, we hypothesized that oxidative stress is a factor that triggers the up-regulation of mitochondrial biogenesis genes in experimental animals chronically fed EtOH. If our hypothesis is correct, then the co-administration of EtOH together with antioxidants should prevent both EtOH-induced oxidative stress and up-regulation of mitochondrial biogenesis genes. To test our hypothesis, rats were administered EtOH intragastrically for 150 days, in the absence or presence of the antioxidant *N*-acetylcysteine (NAC) at 1.7 g/kg/d; control rats were administered an isocaloric diet. At the end of the treatment period, mitochondrial biogenesis and oxidative stress markers were evaluated in extracted livers.

MATERIALS AND METHODS

Animals and Experimental Design

Four groups of male Sprague–Dawley rats weighing 350 g had an intragastric cannula surgically implanted and were infused (166 kcal/kg$^{3/4}$/d) with isocaloric liquid diets using total enteral nutrition (TEN) as described previously (Ronis et al., 2011). The control group (Control) received a diet that contained 16% protein (whey peptides), 39% carbohydrate (dextrose and maltodextrin), and 45% fat (corn oil) together with National Research Council-recommended levels of vitamins and minerals (Ronis et al., 2011). In the EtOH-treated group (EtOH), EtOH substituted isocalorically for carbohydrate calories and the diet composition was 16% protein, 5% carbohydrate, 45% fat, and 34% EtOH calories as described previously (Ronis et al., 2011). In addition, control and EtOH groups were treated with NAC (NAC, EtOH + NAC) at 1.7 g/kg/d added to the diets. After 150 days, the animals were euthanized. The livers were immediately excised, washed in ice-cold saline, rapidly frozen in liquid nitrogen, and stored at −80°C. All the animal studies described above were approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences. All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" at an American Association for Accreditation of Laboratory Animal Care-approved animal facility at Arkansas Children's Hospital Research Institute.

Mitochondrial Homogenates

Frozen liver samples were homogenized (1:10 w/v) with 0.1 M potassium phosphate buffer pH 7.4 in a glass homogenizer at 4°C. After 10 manual strokes with a tight-fitting glass pestle, the homogenate was centrifuged for 10 minutes at 600×*g* and 4°C. The supernatant (labeled liver homogenate) was kept and recentrifuged at 12,000×*g* for 20 minutes at 4°C. The pellet was resuspended in 0.1 ml of 0.1 M potassium phosphate buffer pH 7.4, labeled as mitochondrial homogenate, and after determining protein concentration using the Bradford reagent (Sigma-Aldrich, St. Louis, MO), it was frozen in liquid nitrogen and stored at −80°C until use (Wiedemann et al., 2000). The supernatant of the last centrifugation was labeled as S12 fractions, and after determining protein concentration using the Bradford reagent (Sigma-Aldrich), it was frozen in liquid nitrogen and stored at −80°C until use. To verify that the mitochondrial homogenate obtained was enriched in mitochondrial membrane components, an immunoblot for ATP synthase (F1-ATPase) alpha subunit (ATP5A; a mitochondrial marker) was performed on S12 fractions and mitochondrial homogenates as described below. Enrichment of mitochondrial membranes was verified by the high density of the immunoreactive band in mitochondrial homogenates with respect to S12 fractions, which represented 95% of the total density of the ATP5A bands (Fig. 1*A*). In addition, cytochrome c oxidase activity (an enzymatic activity associated with the inner mitochondrial membrane, determined as described below) was undetectable in S12 fractions, another indication that mitochondrial homogenates were highly enriched in mitochondrial membranes (Fig. 1*B*). Cross-contamination between the S12 fraction and mitochondrial membranes in both control and EtOH samples (with and without NAC) was low, as shown by the ratio of the band intensity of the mitochondrial marker ATP5A in the mitochondrial fraction compared with ATP5A in the S12 fraction (range of 11.9 to 18.5), and the ratio of the band intensity of the cytosolic marker lactate dehydrogenase (LDH) in the S12 fraction compared with lactate dehydrogenase (LDH) in the mitochondrial fraction (range of 2.4 to 4.0; Fig. 1*C*).

DNA Extraction

Genomic DNA was extracted from frozen liver tissue using the DNEasy kit (Qiagen, Valencia, CA) following the manufacturer's instructions. DNA concentration was determined by fluorescence labeling using the Pico Green dsDNA kit (Life Technologies, Grand Island, NY).

RNA Extraction

Total RNA was isolated using Trizol reagent (Life Technologies) and further purified with a Qiagen RNeasy kit (Qiagen; Steffensen et al., 2004). RNA concentration was determined by UV absorbance at 260 nm using an extinction coefficient in TE buffer (10 mM Tris-HCl, pH 7.5, EDTA 1 mM) of 0.025/(μg/ml)/cm. Purity was evaluated by the ratio of absorbance at 260 and 280 nm, and 260 and 230 nm (always ≥1.8).

mtDNA Content

The relative amount of mtDNA in rat liver was determined using quantitative real-time polymerase chain reaction (PCR) as described by Tchaparian and colleagues (2010).

mRNA Expression

The expression of mitochondrial biogenesis genes was determined by quantification of specific mRNAs by real-time reverse-transcriptase (RT) PCR. Real-time RT-PCR was performed by a 1-step method with 10 ng of total RNA using QuantiFast SYBR Green RT-PCR Kit (Qiagen). Quantitect primers (Qiagen) were used for reverse transcription and PCR amplification. The expression levels of each gene (PGC-1α and TFAM) are presented as values normalized against β-actin as housekeeping gene.

Mitochondrial Mass

The content of mitochondria (mitochondrial mass) of the livers was calculated from the ratios of cytochrome oxidase activities in liver homogenates and in mitochondrial homogenates (Navarro et al., 2009). Alternatively, the activity in liver homogenates of the mitochondrial enzyme citrate synthase was used as an index of mitochondrial mass, evaluated as described by Kirby and colleagues (2007).

Respiratory Chain Complex Activities

Respiratory chain complex I, II, and IV activities were determined in mitochondrial homogenates subjected to 3 rounds of freeze/thaw according to Kirby and colleagues (2007). Complex I activity was determined by analyzing the oxidation of NADH to NAD^+ in mitochondrial homogenates using an immunocapture complex I enzyme activity assay (MitoSciences MS141; Abcam, Cambridge, MA). Complex II activity was measured as succinate: ubiquinone$_1$ oxidoreductase linked to the artificial electron acceptor 2,6 dichlorophenolindophenol as described in Kirby and colleagues (2007). Cytochrome c oxidase activity (complex IV) was measured by following the oxidation of ferrocytochrome c (Kirby et al., 2007).

Western Blots

Western blots were performed on liver lysates or mitochondrial homogenates. To prepare liver lysates for Western blot, frozen liver samples were homogenized in RIPA buffer (Sigma-Aldrich) and incubated on ice for 15 minutes. Lysates were cleared by centrifugation at 12,000×*g* and 4°C for 15 minutes. After determining protein concentration using the Bradford reagent (Sigma-Aldrich), 50 μg of protein from liver lysates or mitochondrial homogenates were diluted in TE buffer and then mixed with Laemmli buffer, heated at 95°C for 10 minutes, and separated by SDS-PAGE. After transferring the separated proteins to nitrocellulose membranes, membranes were immunoblotted with specific antibodies against PGC-1α (Santa Cruz Biotechnology, Dallas, TX;, sc13067), TFAM (Santa Cruz Biotechnology; sc23588), SDHA (Abcam; ab14715), MTCO1 (Abcam; ab14705), NDUFS3 (Abcam; ab14711), LDH (Santa Cruz Biotechnology; sc33781), complex IV subunit IV (Abcam; ab14744), p38 MAPK (p38; Santa Cruz Biotechnology; sc535), or phosphorylated p38 MAPK (pp38; Santa Cruz Biotechnology; sc7973). The membranes were then incubated with the corresponding secondary horseradish-conjugated antibody and immunoreactive bands were visualized by chemiluminescence (Chemiglow; Protein Simple, Santa Clara, CA). Membranes were stripped once and reprobed with antibodies against beta actin (Santa Cruz Biotechnology; sc81178) or ATP5A (Abcam; ab14748) as loading controls for liver lysates or mitochondrial homogenates, respectively. ATP5A was used as loading control for mitochondrial homogenates, because its expression was constant throughout the different treatments (verified using Ponceau S as loading control; data not shown). The density of protein bands was quantitatively analyzed

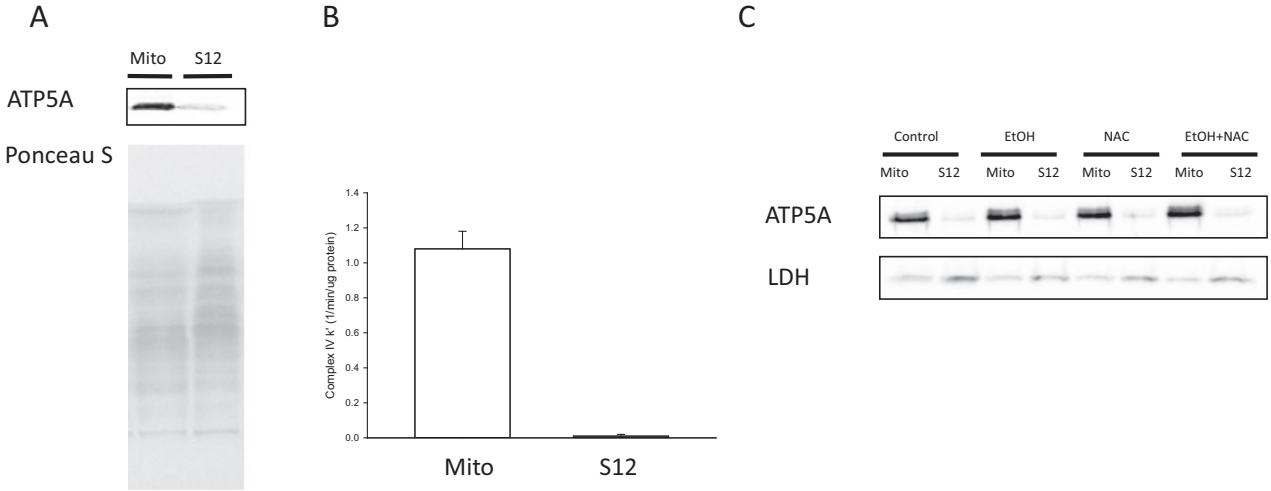

Fig. 1. Distribution of subcellular markers between mitochondrial homogenates and S12 fractions. Mitochondrial homogenates and S12 fractions were isolated from livers from rats administered ethanol (EtOH), EtOH + *N*-acetylcysteine (NAC; EtOH + NAC), NAC alone (NAC) or a control isocaloric diet (Control) by total enteral nutrition as described in Materials and Methods. (**A**) Control samples were used to: assess protein levels of ATP5A (a mitochondrial marker) by Western blot with Ponceau S as loading control; (**B**) Control samples were used to assess the enzymatic activity of cytochrome c oxidase (complex IV). (**C**) Control, EtOH, NAC, and EtOH + NAC samples were used to assess protein levels of ATP5A by Western blot and, after stripping the nitrocellulose membrane, lactate dehydrogenase (LDH; a cytosolic marker). Shown are the representative results of 3 independent experiments.

by ImageJ software (NIH, Bethesda, MD) and expressed as a relative ratio against the loading control.

Glutathione Determination

Frozen liver samples were homogenized (1:10 w/v) with 0.1 M potassium phosphate buffer pH 7.4 in a glass homogenizer at 4°C. After 10 manual strokes with a tight-fitting glass pestle, the homogenate was centrifuged for 10 minutes at 600×*g* and 4°C. After determining protein concentration using the Bradford reagent, the supernatant was treated with 10% trichloroacetic acid (2:1 v/v) to extract tissue glutathione. After centrifuging the mixture at 14,500×*g* for 10 minutes at 4°C to remove denatured proteins, reduced glutathione (GSH) and glutathione disulfide (GSSG) were determined in the supernatant by the recycling enzymatic method of Tietze as described in Mari and Cederbaum (2000). Mitochondrial glutathione was not determined because of the lack of structural integrity of the isolated mitochondrial fraction.

Protein Carbonyls

Protein carbonyls were quantified in mitochondrial homogenates and S12 fractions by ELISA after derivatization of the samples with dinitrophenylhydrazine, using the Biocell PC kit (Biocell Corp., Auckland, NZ).

Biochemical Markers of Mitochondrial Oxidative Stress

Biochemical markers of mitochondrial oxidative stress included markers of oxidative damage to mitochondrial proteins (protein carbonyls), phospholipids (thiobarbituric acid-reactive substances [TBARS]), and DNA (lesions that block the progression of the thermostable DNA polymerase in long quantitative PCR [qPCR]). Protein carbonyls were quantified as described above. TBARS was determined as previously described (Caro and Cederbaum, 2001). Oxidative damage to mtDNA was determined by long qPCR as described in Santos and colleagues (2002).

Statistical Analysis

Data are expressed as mean ± SE of the mean from 3 to 9 independent experiments run in duplicate. Statistical differences were analyzed by 2-way analysis of variance (ANOVA); when a significant interaction was detected, differences among individual means were assessed with Tukey's multiple comparison test. If no significant interaction was detected, a 1-way ANOVA was performed. SigmaPlot (Systat Software, San Jose, CA) was used for statistical analysis. A $p < 0.05$ was considered as statistically significant.

RESULTS

Effects of Alcohol and NAC on Body Composition and Hepatic Pathology

Comprehensive analysis of the development of liver pathology in this experiment after chronic EtOH consumption with or without co-administration of NAC in the TEN model has been previously published (Ronis et al., 2011; Setshedi et al., 2011). Blood EtOH values were 196 ± 35 and 173 ± 33 mg/dl in the EtOH and EtOH + NAC groups, respectively. Body weight gain was significantly suppressed by EtOH treatment in both groups, but EtOH treatment had no effects on adiposity in this model (Ronis et al., 2011). As expected, EtOH treatment significantly increased liver/body weight ratio equally in both treatment groups, significantly increased serum ALT values, a marker of necrotic injury, and resulted in alcoholic steatohepatitis including development of fibrosis. NAC treatment failed to reverse development of steatosis but prevented EtOH-induced necroinflammatory injury and had additive effects with EtOH to induce hepatocyte proliferation (Ronis et al., 2011). Liver injury was accompanied by impaired hepatic insulin-IGF-1 signaling which was not restored by NAC treatment (Setshedi et al., 2011).

Effect of NAC on EtOH-Induced Hepatic Oxidative Stress

NAC was used as an antioxidant because it can act as a precursor of GSH and as a direct scavenger of ROS (Sadowska et al., 2006). To determine the extent of the antioxidant activity of NAC in the TEN model, the redox state of the GSH/GSSG pair was evaluated. The redox state of the GSH/GSSG pair is an index of the cellular redox state in many tissues including liver and is thus frequently used as an indicator of oxidative stress (Han et al., 2006). The redox state of the GSH/GSSG pair is determined by measuring the concentrations and the ratios of the main interconvertible reduced (GSH) and oxidized (GSSG) forms of glutathione (Rebrin et al., 2003). The hepatic level of total glutathione and the GSH/GSSG ratio were significantly lower in EtOH-treated rats than in control rats (Table 1), reflecting hepatic oxidative stress conditions induced by chronic EtOH. Cotreatment with

Table 1. Effect of Ethanol (EtOH) and *N*-Acetylcysteine (NAC) on Hepatic Oxidative Stress

Group	Total glutathione (nmol/mg prot)	GSH/GSSG	Protein carbonyls (nmol/mg prot)
Control	49 ± 3	55 ± 6	0.11 ± 0.04
EtOH	35 ± 5*	37 ± 5*	0.34 ± 0.05*
NAC	54 ± 4	60 ± 7	0.18 ± 0.03
EtOH + NAC	48 ± 3#	63 ± 7#	0.21 ± 0.03*#
ANOVA (*p*-values)[a]			
EtOH effect	0.010	0.040	<0.001
NAC effect	0.014	0.002	0.198
Interaction (EtOH × NAC)	0.405	0.383	0.002

*Significantly different ($p < 0.05$) with respect to the control group.

#Significantly different ($p < 0.05$) with respect to the same treatment, but in the absence of NAC.

[a]Results from 2-way ANOVA with EtOH effect (absent vs. present), NAC effect (absent vs. present), and interaction (EtOH × NAC).

Rats were administered EtOH by total enteral nutrition for 150 days, in the absence (EtOH group) or presence (EtOH + NAC group) of the antioxidant NAC at 1.7 g/kg/d; control rats were administered an isocaloric diet in the absence (Control group) or presence (NAC group) of NAC. After extracting liver glutathione with trichloroacetic acid, the ratio between reduced glutathione (GSH) and glutathione disulfide (GSSG), and total glutathione (GSH + GSSG) levels were assessed using an enzymatic recycling method as described in Materials and Methods. The degree of protein oxidation assessed as protein carbonyls was evaluated in S12 liver fractions.

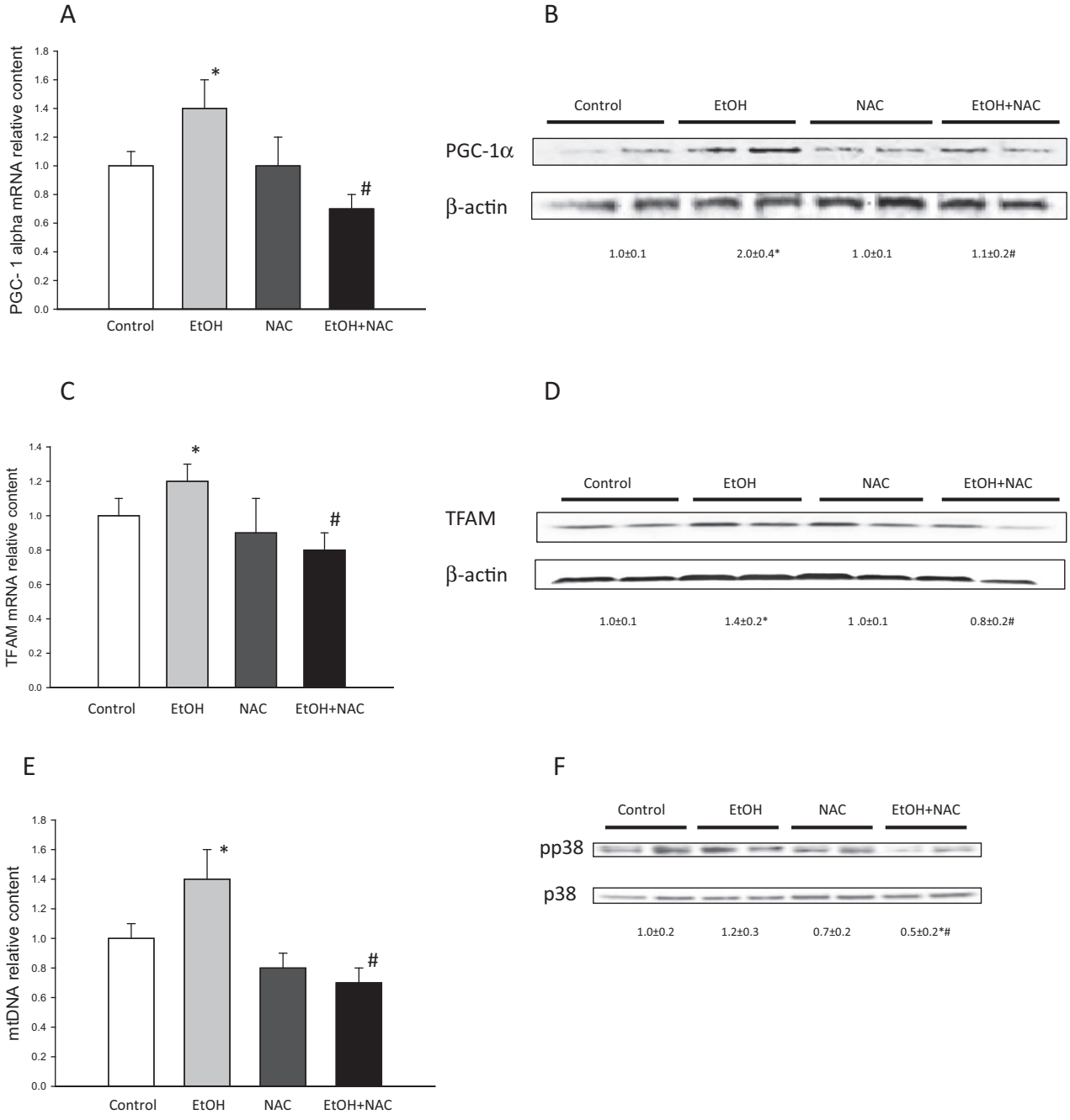

Fig. 2. Effect of ethanol (EtOH) and *N*-acetylcysteine (NAC) on biochemical markers of mitochondrial biogenesis. Liver RNA, liver lysates, and genomic DNA were isolated from rats administered EtOH, EtOH and NAC (EtOH + NAC), NAC alone (NAC) or a control isocaloric diet (Control) by total enteral nutrition as described in Materials and Methods. (**A**) PGC-1α mRNA levels were quantified by real-time reverse-transcriptase PCR using specific primers, normalized to β-actin and expressed as fold induction over the control group; (**B**) PGC-1α protein levels were quantified by Western blot using specific antibodies, using beta actin as loading control. Numbers below the blots represent the relative density of the PGC-1α protein bands normalized to β-actin and expressed as fold induction over the control group; (**C**) mitochondrial transcription factor A (TFAM) mRNA levels were quantified by real-time reverse-transcriptase PCR using specific primers, normalized to β-actin and expressed as fold induction over the control group; (**D**) TFAM protein levels were quantified by Western blot using specific antibodies, using beta actin as loading control. Numbers below the blots represent the relative density of the PGC-1α protein bands normalized to β-actin and expressed as fold induction over the control group; (**E**) Mitochondrial DNA was quantified by real-time PCR using specific primers, normalized to a nuclear DNA gene and expressed as fold induction over the control group. (**F**) Activation of p38 MAPK was assessed by evaluating protein levels of phosphorylated p38 (pp38) and total p38 (p38) MAPK by Western blot using specific antibodies. Numbers below the blots represent the ratio of pp38 MAPK/p38 MAPK. Data are expressed as mean $\pm$ standard error of the mean from 4 independent experiments. Shown are representative Western blots. Two-way ANOVA analysis for every panel indicated a nonsignificant effect of EtOH ($p > 0.05$), a significant effect of NAC ($p < 0.050$) and a nonsignificant (EtOH $\times$ NAC) interaction ($p > 0.05$). *Significantly different ($p < 0.05$) with respect to the control group. #Significantly different ($p < 0.05$) with respect to the same treatment, but in the absence of NAC.

EtOH and NAC prevented the effects of EtOH on total glutathione and the GSH/GSSG ratio (Table 1), reflecting a high antioxidant activity of NAC in the TEN model. Oxidative damage to liver proteins was evaluated in S12 fractions by quantifying protein carbonyls. Protein carbonyl content increased in EtOH-treated rats with respect to control rats (Table 1); cotreatment with EtOH and NAC inhibited the increase in protein carbonyls caused by EtOH, showing that NAC prevented protein oxidation in liver S12 fractions.

Effect of NAC on the EtOH-Induced Up-Regulation of Mitochondrial Biogenesis Genes

PGC-1α is the transcriptional coactivator that controls the expression of genes involved in mitochondrial biogenesis (Irrcher et al., 2009). Because ROS can induce the transcriptional activation of PGC-1α (Irrcher et al., 2009), the effect of EtOH (in the presence or absence of NAC) on PGC-1α mRNA levels was evaluated. PGC-1α mRNA content significantly increased in livers from EtOH-treated rats with respect to control rats; this effect was prevented by cotreatment with EtOH and NAC (Fig. 2*A*). The expression of PGC-1α was evaluated at the protein level by Western blotting. Quantification of the bands by densitometry showed that the PGC-1α content increased in EtOH-treated rats and that this increase was prevented by cotreatment with EtOH and NAC (Fig. 2*B*). No significant differences in β-actin protein levels were observed between control and treated samples (data not shown).

PGC-1α induces mitochondrial biogenesis by activating transcription factors including NRF1 and NRF2, which in turn increase the expression of mitochondrial proteins including TFAM, which is essential for mtDNA replication and transcription (Hock and Kralli, 2009). It was important to evaluate if the up-regulation of PGC-1α induced the expression of downstream target genes such as TFAM. The mRNA (Fig. 2*C*) and protein (Fig. 2*D*) levels of TFAM increased in livers from rats treated chronically with EtOH. These effects were prevented by cotreatment with EtOH and NAC (Fig. 2*C*, *D*). Because TFAM protein levels directly regulate mtDNA copy number in vivo (Ekstrand et al., 2004), mtDNA content was evaluated to determine the consequences of TFAM overexpression in rats chronically fed EtOH. mtDNA content increased in livers from EtOH-treated rats (Fig. 2*E*); this effect was prevented by cotreatment with EtOH and NAC (Fig. 2*E*).

In the liver and other tissues, activation of PGC-1α gene expression is promoted by activation of the p38 MAPK pathway (Fernandez-Marcos and Auwerx, 2011). Because the p38 MAPK pathway is activated by oxidative stress (Son et al., 2011), the effect of EtOH (in the presence or absence of NAC) on p38 MAPK activation was evaluated. Chronic EtOH treatment produced a nonsignificant increase in p38 activation with respect to controls; co-administration of EtOH and NAC produced a significant decrease in p38 activation with respect to controls (Fig. 2*F*).

Table 2. Effect of Ethanol (EtOH) and *N*-Acetylcysteine (NAC) on Liver Mitochondrial Mass Content

Group	Cytochrome oxidase: Liver homogenate (1/s/g organ)	Cytochrome oxidase: Mitochondrial homogenate (1/s/mg protein)	Mitochondrial mass (mg protein/g organ)
Control	583 ± 83	10.0 ± 1.2	62.8 ± 10.0
EtOH	148 ± 15*	5.7 ± 1.2*	30.5 ± 4.7*
NAC	524 ± 62	8.9 ± 1.0	60.2 ± 5.1
EtOH + NAC	183 ± 17*	5.3 ± 0.7*	37.0 ± 4.6*
ANOVA (*p*-values)[a]			
EtOH effect	<0.001	<0.001	<0.001
NAC effect	0.689	0.078	0.674
Interaction (EtOH × NAC)	0.150	0.867	0.351

*Significantly different ($p < 0.05$) with respect to the control group.
[a]Results from 2-way ANOVA with EtOH effect (absent vs. present), NAC effect (absent vs. present), and interaction (EtOH × NAC).
Rats were administered EtOH by total enteral nutrition for 150 days, in the absence (EtOH group) or presence (EtOH + NAC group) of the antioxidant NAC at 1.7 g/kg/d; control rats were administered an isocaloric diet in the absence of NAC (Control group). Mitochondrial mass was calculated from the ratios of cytochrome oxidase activity in liver homogenates and in mitochondrial homogenates isolated from the same liver samples.

Table 3. Effect of Ethanol (EtOH) and *N*-Acetylcysteine (NAC) on Citrate Synthase Activity

Group	Citrate synthase (nmol/min/mg protein)
Control	32 ± 3
EtOH	14 ± 3*
NAC	27 ± 2
EtOH + NAC	19 ± 2*
ANOVA (*p*-values)[a]	
EtOH effect	<0.001
NAC effect	1.000
Interaction (EtOH × NAC)	0.130

*Significantly different ($p < 0.05$) with respect to the control group.
[a]Results from 2-way ANOVA with EtOH effect (absent vs. present), NAC effect (absent vs. present), and interaction (EtOH × NAC).
Rats were administered EtOH by total enteral nutrition for 150 days, in the absence (EtOH group) or presence (EtOH + NAC group) of the antioxidant NAC at 1.7 g/kg/d; control rats were administered an isocaloric diet in the absence of NAC (Control group). Citrate synthase activity in liver homogenates was determined as an index of mitochondrial mass.

The up-regulation of the mitochondrial biogenesis program can produce an increase in mitochondrial mass and augmented bioenergetic capacity (Han et al., 2012). Liver mitochondrial mass was determined to evaluate if variations in mitochondrial mass is one of the consequences of the changes in expression of mitochondrial biogenesis genes in rats administered EtOH by TEN in the absence or presence of NAC. Mitochondrial mass decreased in livers from rats treated chronically with EtOH; cotreatment with EtOH and NAC did not produce a significant change in liver mitochondrial mass with respect to rats treated with EtOH

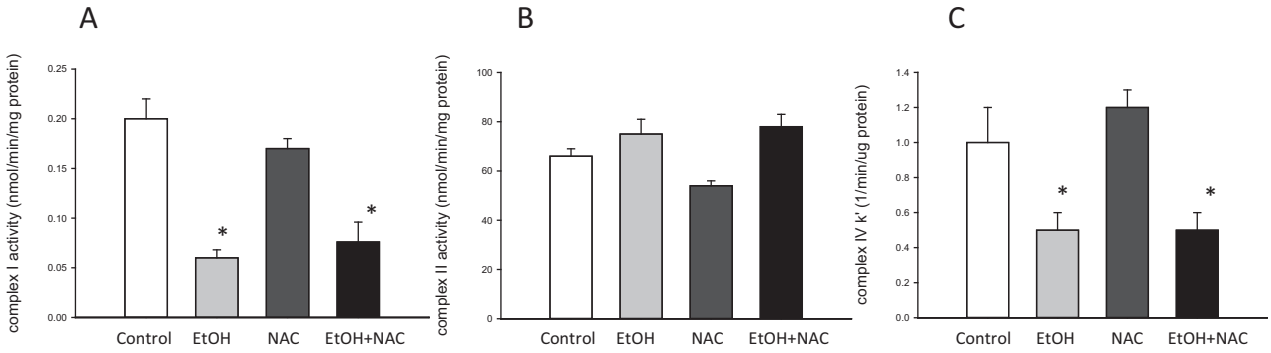

Fig. 3. Effect of ethanol (EtOH) and *N*-acetylcysteine (NAC) on the enzymatic activity of mitochondrial respiratory complexes. Mitochondrial homogenates were isolated from rats administered EtOH, EtOH and NAC (EtOH + NAC), NAC alone (NAC) or a control isocaloric diet (Control) by total enteral nutrition as described in Materials and Methods. The enzymatic activity of individual complexes I (**A**), II (**B**), and IV (**C**) was assessed as described in Materials and Methods. Data are expressed as mean ± SE of the mean from 6 independent experiments. Two-way ANOVA analysis for every panel indicated a significant effect of EtOH ($p < 0.05$) but not of NAC ($p > 0.050$) and a nonsignificant (EtOH × NAC) interaction ($p > 0.05$). *Significantly different ($p < 0.05$) with respect to the control group.

alone (Table 2). Mitochondrial mass was also estimated by measuring in liver homogenates the activity of the mitochondrial enzyme citrate synthase. Citrate synthase activity decreased in livers from rats treated chronically with EtOH; cotreatment with EtOH and NAC did not produce a significant change in citrate synthase activity with respect to rats treated with EtOH alone (Table 3). In order to evaluate the extent to which variations in the expression of mitochondrial biogenesis genes in rats fed EtOH chronically affected mitochondrial respiration, the activity of mitochondrial respiratory complexes I, II, and IV was determined. Prolonged EtOH administration significantly decreased the activity of complex I and IV of the respiratory chain, but did not affect the activity of complex II (Fig. 3*A–C*). Co-administration of EtOH and NAC did not prevent the changes caused by administration of EtOH alone (Fig. 3*A–C*). Because protein levels of respiratory complexes play an important role in determining the respiration rate of mitochondria (Han et al., 2012), the protein levels of essential subunits for the function of respiratory complexes were determined. Prolonged EtOH administration to rats significantly decreased the protein levels of complex I (nuclear-encoded NDUFS3 subunit) and IV (both nuclear-encoded IV subunit and mitochondrial-encoded MTCO1 subunit), but did not affect protein levels of complex II (nuclear-encoded SDHA subunit). Co-administration of EtOH and NAC did not prevent the changes in protein levels of respiratory complex subunits caused by administration of EtOH alone (Fig. 4).

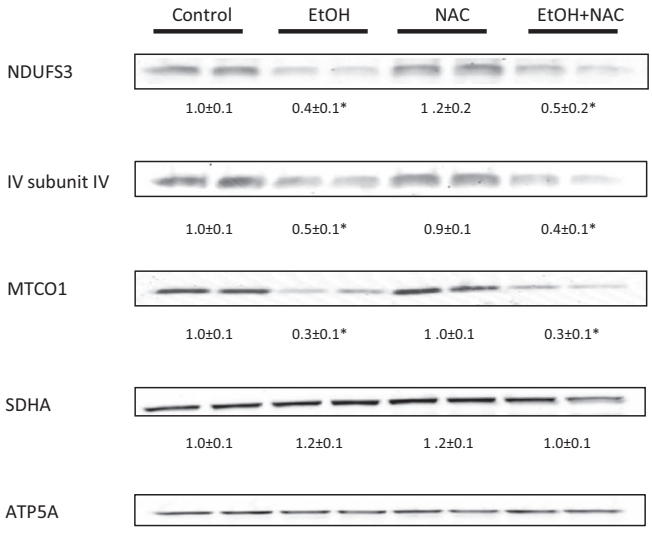

Fig. 4. Effect of ethanol (EtOH) and *N*-acetylcysteine (NAC) on the protein content of mitochondrial respiratory complexes. Mitochondrial homogenates were isolated from rats administered EtOH, EtOH and NAC (EtOH + NAC), NAC alone (NAC) or a control isocaloric diet (Control) by total enteral nutrition as described in Materials and Methods. Protein levels of NDUFS3, IV subunit IV, MTCO1, and SDHA were quantified by Western blot using specific antibodies, using ATP5A as loading control. Numbers below the blots represent the relative density of the specific protein bands normalized to ATP5A and expressed as fold induction over the control group. Data are expressed as mean ± SE of the mean from 4 independent experiments. Shown are representative Western blots. Two-way ANOVA analysis for every panel except SDHA indicated a significant effect of EtOH ($p < 0.05$). Two-way ANOVA analysis for every panel indicated a nonsignificant effect of NAC ($p > 0.050$) and a nonsignificant (EtOH × NAC) interaction ($p > 0.05$). *Significantly different ($p < 0.05$) with respect to the control group.

Effect of NAC on EtOH-Induced Mitochondrial Oxidative Damage

The fact that NAC can increase cytosolic glutathione in hepatocytes from rats chronically fed EtOH, but does not increase the mitochondrial glutathione pool (Garcia-Ruiz et al., 1995), suggests that NAC might be a less effective antioxidant at the mitochondrial level. Therefore, the effect of NAC on specifically mitochondrial oxidative stress was evaluated. Mitochondrial oxidative stress markers (including protein carbonyls, TBARS, and mtDNA damage) were higher in livers from rats chronically fed EtOH than from control rats (Table 4); co-administration of EtOH and NAC did not change the levels of mitochondrial oxidative stress markers with respect to EtOH alone (Table 4).

Table 4. Effect of Ethanol (EtOH) and *N*-Acetylcysteine (NAC) on Liver Mitochondrial Oxidative Stress

Group	Protein carbonyls (nmol/mg protein)	TBARS (nmol/mg protein)	mtDNA integrity (relative ratio)
Control	0.17 ± 0.03	0.77 ± 0.05	1.0 ± 0.1
EtOH	0.40 ± 0.07*	1.7 ± 0.1*	0.55 ± 0.09*
NAC	0.25 ± 0.02	0.88 ± 0.07	1.2 ± 0.3
EtOH + NAC	0.53 ± 0.05*	1.9 ± 0.1*	0.46 ± 0.06*
ANOVA (*p*-values)[a]			
EtOH effect	0.019	<0.001	0.004
NAC effect	0.385	0.263	0.740
Interaction (EtOH × NAC)	0.879	0.535	0.411

*Significantly different ($p < 0.05$) with respect to the control group.

[a]Results from 2-way ANOVA with EtOH effect (absent vs. present), NAC effect (absent vs. present), and interaction (EtOH × NAC).

Rats were administered EtOH by total enteral nutrition for 150 days, in the absence (EtOH group) or presence (EtOH + NAC group) of the antioxidant NAC at 1.7 g/kg/d; control rats were administered an isocaloric diet in the absence (Control group) or presence (NAC group) of NAC. After isolating liver mitochondrial homogenates, mitochondrial protein and phospholipid oxidative damage was evaluated as concentration of protein carbonyls and TBARS, respectively, expressed in nmol/mg protein units. Mitochondrial DNA damage was evaluated in liver genomic DNA by quantifying mitochondrial lesions that block the progression of the thermostable DNA polymerase in long qPCR and expressed as relative mtDNA integrity.

DISCUSSION

Two lines of evidence suggest that the up-regulation of mitochondrial biogenesis genes by chronic EtOH is induced by ROS: first, there is a positive association between hepatic oxidative stress by chronic EtOH and up-regulation of mitochondrial biogenesis genes; and second, an antioxidant such as NAC blocks both the EtOH-induced hepatic oxidative stress and the up-regulation of mitochondrial biogenesis genes. Other oxidative stress models in different mammalian systems also induced up-regulation of mitochondrial biogenesis genes which could be blocked by antioxidants, suggesting that up-regulation of mitochondrial biogenesis is a general response triggered by oxidative stress in mammalian cells. For example: (i) exercise training up-regulated PGC-1α, NRF1, and TFAM in rat skeletal muscle, an effect that was blocked by administration of vitamin C (Gomez-Cabrera et al., 2008); (ii) C2C12 muscle cells exposed to H_2O_2 in vitro up-regulated PGC-1α, an effect prevented by NAC (Irrcher et al., 2009); (iii) short-term exercise up-regulated PGC-1α in human skeletal muscle, an effect prevented by oral administration of a combination of vitamin C and vitamin E (Ristow et al., 2009); and (iv) prolonged (7 weeks) EtOH administration in the drinking water increased liver PGC-1α in wild-type mice, an effect partially prevented in transgenic manganese superoxide dismutase (MnSOD) mice (Larosche et al., 2009). Future experiments using other antioxidants will help to identify the nature of the oxidant species that up-regulate mitochondrial biogenesis in chronic alcoholism.

The main mechanism of antioxidant action of NAC is to promote the synthesis of hepatic GSH (Han et al., 2006). NAC promotes GSH synthesis because NAC is a precursor of cysteine, and the availability of cysteine is the rate-limiting step in GSH synthesis (Burgunder et al., 1989). GSH is synthesized in the cytosol and transported into mitochondria, where it accumulates at a fraction representing around 10% of total hepatic GSH (Han et al., 2006). Previous research has shown that despite significantly increasing the cytosolic GSH pool in livers from EtOH-fed rats, NAC treatment did not result in a significant increase of the mitochondrial GSH pool (Garcia-Ruiz et al., 1995). The inability of NAC to increase the mitochondrial GSH pool in livers from EtOH-fed rats may be related to the fact that chronic EtOH impairs the transport and accumulation of cytosolic GSH into the mitochondrial matrix (Fernandez et al., 2013). These observations suggest that NAC, while being an extra-mitochondrial hepatic antioxidant due to its ability to increase extra-mitochondrial hepatic GSH, is unable to counteract mitochondrial oxidative stress in hepatocytes exposed to EtOH. The results presented in our work confirm this hypothesis because the EtOH-induced oxidative damage to mitochondrial biomolecules was not prevented by co-administration of NAC, in spite of the fact of increasing total GSH levels and prevention of extra-mitochondrial protein oxidation. Because mitochondrial biogenesis signaling induced in rats treated with EtOH was effectively blocked by NAC in our work, it is probable that extra-mitochondrial effects of ROS result in a signaling cascade that leads to biogenesis of the organelle, as suggested by Irrcher and colleagues (2009).

The gene expression of PGC-1α is known to be regulated by ROS (Irrcher et al., 2009). However, the molecular mechanisms by which ROS regulate PGC-1 α transcription in liver are not completely understood. The most important signaling pathways that link ROS with PGC-1 α gene transcription are CaMKIV, AMPK, and p38 (Irrcher et al., 2009). The observation that NAC decreased p38 MAPK activation in EtOH-treated rats suggests that the inhibition of the up-regulation of mitochondrial biogenesis genes caused by NAC could be partially mediated by p38 MAPK inactivation. The possible role of other mediators in mitochondrial biogenesis induced by chronic EtOH in rats is currently being investigated. Although increased gene transcription and protein expression likely increases the coactivator activity of PGC-1 α, this activity is also regulated transcriptionally and post-transcriptionally by alternative promoter usage and alternative splicing of the primary transcript (Ruas et al., 2012), respectively, and post translationally by phosphorylation and acetylation (Fernandez-Marcos and Auwerx, 2011). Therefore, more data are needed to completely characterize the effect of chronic EtOH on PGC-1 α coactivator activity.

Mitochondrial biogenesis is a program directed toward compensating against mitochondrial oxidative damage and maintaining mitochondrial mass (Piantadosi, 2008). In this

respect, chronic EtOH administration by intragastric infusion in mice has been shown to induce mitochondrial biogenesis genes and increase mitochondrial mass (Han et al., 2012). However, in our work, up-regulation of mitochondrial biogenesis genes caused by chronic EtOH administration by TEN in rat liver occurred in the context of decreased mitochondrial mass (evaluated both as mitochondrial protein per gram of liver and citrate synthase activity in liver homogenates). This apparent contradiction can be explained by the fact that the steady state of mitochondrial mass is determined by the balance between mitochondrial biogenesis and mitochondrial degradation via autophagy (mitophagy; Berman et al., 2009). An imbalanced condition where mitochondrial degradation rate is higher than biogenesis will induce a decrease in mitochondrial mass (Berman et al., 2009). Therefore, our results suggest that in rat liver, mitochondrial oxidative stress induced by chronic EtOH produces mitochondrial degradation that is not adequately balanced by a concomitant increase in mitochondrial biogenesis, resulting in lower mitochondrial mass. The differential effect of chronic EtOH in rats and mice could be ascribed to different species, EtOH administration protocol, dose, and exposure time. The balance between mitochondrial biogenesis and degradation may also be affected by diet composition. The TEN model of chronic alcohol administration utilized in the current studies has a high level of dietary polyunsaturated fats (45%) and a very low level of carbohydrate (5% of total calories in the 34% EtOH calorie diet as EtOH isocalorically replaced carbohydrate calories). We have previously shown that higher levels of dietary carbohydrate result in protection against progression of alcoholic liver disease (ALD) from simple steatosis to steatohepatitis (Korourian et al., 1999; Ronis et al., 2004). One possible mechanism for this protection may involve availability of energy for repair of mitochondrial injury. Further experiments to examine the effects of dietary carbohydrate content on the balance between alcohol-mediated mitochondrial biogenesis and degradation are in progress.

Mitochondrial oxidative stress could also contribute to decreased mitochondrial function at the level of the oxidative phosphorylation system. Protein oxidation and inactivation, and mtDNA oxidative damage could contribute to a decline in the levels of active respiratory complexes. The protein levels of both nuclear-encoded (NDUFS3, complex IV subunit VIIaHL) and mitochondrial-encoded (MTCO1) subunits of respiratory chain complexes were decreased by chronic EtOH, confirming literature results (Venkatraman et al., 2004). Protein levels of mitochondrial-encoded subunits could have decreased because of lower transcription caused by mtDNA oxidative damage, or translation caused by oxidative damage to mitochondrial ribosomes (Bailey et al., 2006). Protein levels of nuclear-encoded subunits could have decreased because of decreased import or increased rates of protein degradation following import during conditions of EtOH-induced oxidative stress in mitochondria, as suggested by Venkatraman and colleagues (2004). In our model, complex II protein and activity levels were not affected by EtOH, confirming that complex II is resistant to oxidative stress (Bansal et al., 2012). In our work, NAC did not prevent the impairment in mitochondrial functionality caused by chronic EtOH probably because of its lack of effect on mitochondrial oxidative damage. As suggested by other authors, antioxidants that are capable of preventing mitochondrial oxidative stress should therefore be more effective in preventing mitochondrial functional impairment. For example, S-adenosyl methionine increased mitochondrial glutathione in rats fed EtOH chronically and preserved mitochondrial function (Garcia-Ruiz et al., 1995); mitochondrial overexpression of MnSOD decreased mitochondrial oxidative stress and preserved mitochondrial functionality in mice after acute EtOH administration (Larosche et al., 2010); betaine supplementation decreased mitochondrial nitric oxide and alterations to mitochondrial respiratory proteome caused by chronic EtOH feeding in rats (Kharbanda et al., 2012); and mitochondria-targeted ubiquinone prevented the decrease in complex I 30 kDa subunit and complex IV subunit IV protein levels in rats chronically fed EtOH (Chacko et al., 2011).

Because of its antioxidant action, its excellent tolerance and safety profile, NAC is a potential therapeutic agent in the treatment of ALD (Moreno et al., 2010). In experimental animals fed EtOH for prolonged periods of time via TEN, NAC was effective in reducing hepatic cytosolic oxidative stress markers, necrosis as measured by ALT release and inflammation (Ronis et al., 2005). However, human trials evaluating NAC have failed to demonstrate a significant beneficial effect in ALD (Moreno et al., 2010; Nguyen-Khac et al., 2011; Stewart et al., 2007). The efficacy of NAC in human trials could be limited by its limited mitochondrial antioxidant activity in vivo, which does not prevent EtOH-induced mitochondrial oxidative damage as shown in this work. Because PGC-1α-induced mitochondrial biogenesis can promote recovery of mitochondrial function after oxidative stress challenges (Rasbach and Schnellmann, 2007b), the effect of NAC of inhibiting mitochondrial biogenesis in the context of sustained mitochondrial damage might prove deleterious for the liver. By inhibiting an adaptive response to oxidative stress such as mitochondrial biogenesis, antioxidant supplementation might actually display negative effects on target organs. For example, in rat muscle exposed to exercise training, antioxidants such as vitamin C and E blocked the adaptive mitochondrial biogenesis program triggered by oxidative stress and decreased endurance capacity, a parameter dependent on mitochondrial content (Gomez-Cabrera et al., 2008; Ristow et al., 2009).

In conclusion, our results suggest that chronic EtOH induces the up-regulation of mitochondrial biogenesis genes in rat liver through signaling pathways triggered by extra-mitochondrial oxidative stress. In spite of the induction of hepatic mitochondrial biogenesis genes by chronic EtOH, mitochondrial mass and function decreased probably in association with mitochondrial oxidative damage.

Our results predict that the effectiveness of NAC as an antioxidant therapy for chronic alcoholism will be limited by its limited antioxidant effects in mitochondria, and its inhibitory effect on mitochondrial biogenesis.

ACKNOWLEDGMENTS

The study was supported by grants from the National Center for Research Resources (P20RR016460), the National Institute of General Medical Sciences (P20 GM103429), and National Institute on Alcohol Abuse and Alcoholism (R01 AA009300) from the National Institutes of Health.

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