Myocardial Oxidative Stress and Toxicity Induced by Acute Ethanol Exposure in Mice

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Alcoholic cardiomyopathy has been known for a long time, but there is little mechanistic insight into this important clinical problem. The present study was undertaken using a mouse model to test the hypothesis that alcohol exposure induces cardiac injury through induction of oxidative stress. Adult female Friend Virius B-type (FVB) mice were treated with ethanol by gavage at a dose of 5 g/kg. Six hours after the treatment, ethanol-induced myocardial injury was observed, as indicated by a significant increase in serum creatine phosphokinase activity, a common biomarker of myocardial injury, and myocardial ultrastructural alterations, predominantly mitochondrial swelling and cristae disarray and reduction in numbers. The myocardial injury was associated with a significant increase in the myocardial lipid peroxidation, determined by measuring thiobarbituric acid reactive substances (TBARS), and a significant increase in protein oxidation as measured by a protein carbonyl content assay. Acute alcohol exposure decreased glutathione (GSH) content in the heart, more so in the mitochondria than in the cytosol. These alcohol-induced myocardial injuries and oxidative stresses were all significantly inhibited by supplementation with N-acetyl-L-cysteine (NAC) prior to alcohol exposure. However, NAC did not affect the rise in blood alcohol concentrations following alcohol exposure. This study thus demonstrates that acute alcohol administration causes myocardial injury through, at least in part, the induction of oxidative stress. A rapid decrease in mitochondrial GSH content may be partially responsible for the observed mitochondrial damage. Exp Biol Med 229:553–559, 2004.

Key words: alcohol; creatine phosphokinase; glutathione; mitochondria; lipid peroxidation; protein oxidation; electron microscopy

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Alcoholic cardiomyopathy, a form of nonischemic dilated cardiomyopathy (1), has been recognized for a long time. Alcoholic cardiomyopathy impairs heart function as well as decreases contractility of the left ventricle and eventually, if untreated, leads to heart failure (2, 3). Several mechanisms have been postulated for the pathogenesis of alcoholic cardiomyopathy, including direct toxic effects of alcohol or its metabolites on mitochondria and sarcoplasmic reticula, disturbance in the intracellular calcium homeostasis, and oxidative stress (4, 5). In addition, alcohol-associated malnutrition and hypertension accelerate the progression of alcoholic cardiomyopathy (6, 7). The risk of developing alcoholic cardiomyopathy is related to both the daily alcohol intake and the duration of drinking, although an individual's susceptibility to this toxic effect varies (8); for instance, women are more vulnerable to the development of alcoholic cardiomyopathy (9).

Oxidative stress derived from alcohol metabolism has been a major focus in the study of alcohol-induced tissue injury (10, 11). Reinke *et al.* (12) have observed that reactive oxygen species (ROS) play an important role in the onset of cardiac toxicity in chronically ethanol-intoxicated animals (12). The pro-oxidant effect of ethanol depends on the induction of a major isoform of the cytochrome P450 family CYP2E1, which has been reported to lead to the formation of ROS (13). Accumulation of ROS such as superoxide, hydrogen peroxide, and hydroxyl radicals along with a compromised antioxidant capacity contribute to excess damage to cellular carbohydrates, proteins, lipids, and nucleic acids (14, 15). Among the endogenous antioxidant systems, reduced glutathione (GSH) plays multiple roles in the detoxification of toxic chemicals (16). Several studies have observed a selective decrease in the GSH content in the mitochondria due to partial inhibition of the specific mitochondrial carrier that translocates GSH from cytosol into the mitochondrial matrix (17). The decrease in mitochondrial GSH may represent an important step in the development of alcohol-induced myocardial oxidative stress and injury.

The GSH levels and the antioxidant enzyme activities in the heart are relatively lower than in other tissues (18). As

a result, heart tissue may be more susceptible to oxidative damage. It has been observed that a single high dose of alcohol exposure caused liver oxidative damage (19, 20). Whether oxidative stress and abnormalities in the cardiac tissue can be produced by a single excessive dose of alcohol has not been addressed. This study was undertaken to examine the effect of acute alcohol exposure on the heart and to evaluate the role of oxidative stress in the alcohol-induced myocardial injury. An acute alcohol exposure mouse model to simulate human binge drinking has been developed. The mice were treated with a single high dose of ethanol by gavage. Six hours after the dosing, the heart was excised and myocardial morphology and serum markers were examined to determine whether acute alcohol exposure causes significant cardiac injury, the involvement of lipid peroxidation and protein oxidation in the cardiac injury, and the effect of acute alcohol on cytosolic and mitochondrial GSH levels in the heart. In addition, the effect of antioxidant N-acetyl-L-cysteine (NAC) supplementation on alcohol-induced myocardial oxidative injury was also examined simultaneously.

Materials and Methods

Animals. In this study, female Friend Virius B-type (FVB) mice 8 weeks of age were used for the consideration of females being more vulnerable to alcohol-induced heart injury. The breeding pairs were obtained from Harlan Bioproducts for Science (Indianapolis, IN), and all of the mice used for experiments were bred in the animal quarters at the University of Louisville Research Resource Center. They were maintained at 22°–23°C on a 12:12-hr light:dark cycle and free access to rodent chow and tap water. The experimental procedures were approved by the Institutional Animal Care and Use Committee, which was certified by the American Association for Accreditation of Laboratory Animal Care.

Ethanol Administration. A binge-drinking mouse model developed by Carson and Pruett (21) was followed for ethanol challenge. This model was designed to achieve blood alcohol levels (BALs), behavioral effects, and physiological changes comparable with human binge drinking. Female FVB mice at 8 weeks of age were divided into four groups: (i) control, (ii) NAC, (iii) ethanol, and (iv) NAC plus ethanol. Mice (Groups 2 and 4) were administered NAC (Calbiochem Corp., La Jolla, CA) at 150 mg/kg by ip injection followed by a 12 hrs of overnight fasting, then administered a second dose of NAC at the same level and by the same route 30 mins prior to a single dose of ethanol at 5 g/kg body weight by gavage (Groups 3 and 4). Control mice received saline for NAC and isocaloric maltose solution for ethanol. At 6 hrs after the ethanol dosing, the mice were anesthetized with sodium pentobarbital (0.05 mg/g body weight). Blood was drawn from the inferior vena cava, and sera were obtained by centrifugation using a serum separator tube. The hearts were rapidly excised, rinsed in saline to remove blood, weighed, frozen immediately in liquid nitrogen, and stored at −80°C for biochemical assays.

Blood Alcohol Assay. BALs were measured using an alcohol dehydrogenase kit (procedure 332-UV; Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions.

Electron Microscopic Examination. To observe ethanol-induced ultrastructural changes by conventional electron microscopy, hearts were fixed *in situ* with Karnovsky fixative (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.4) and postfixed in 1% osmium tetroxide. The procedure has been described previously (22, 23). Ultrathin sections were stained by uranyl acetate and lead citrate and observed with a Philips transmission electron microscope.

Creatine Phosphokinase Activity. Serum creatine phosphokinase (CPK) activity was assayed as described by Oliver (24). A CPK test kit (CK-20; Sigma Chemical Co., St. Louis, MO) based on this method was used. The sera obtained from the blood collected from the inferior vena cava were used for the assay.

Lipid Peroxidation Assay. Ethanol-induced cardiac lipid peroxidation was assessed by measuring thiobarbituric acid–reactive substance (TBARS) concentrations. Heart tissue was homogenized in nine volumes of 1.15% KCl to make a 10% homogenate. To 0.2 ml of the tissue homogenate, 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid, and 2.1 ml of 0.571% thiobarbituric acid were added and vortexed. The reaction mixture was placed in a water bath at 90°C for 70 mins. After cooling on ice, 1.0 ml of distilled water and 5.0 ml of butanol/pyridine mixture (15:1 v/v) were added and vortexed. After centrifugation at 850 *g* for 15 mins, the absorbance of the resulting lower phase was determined at 532 nm. The TBARS concentration was expressed as micromoles TBARS per gram of heart tissue.

Measurement of Protein Carbonyl Concentrations. The oxidized protein was assessed by determination of carbonyl concentrations. Heart tissue was homogenized in nine volumes of 50 mM HEPES buffer (pH 7.2) containing 10 mM KCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5 ml proteinase inhibitor cocktail. To 1.0 ml of homogenate, 4 ml of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 N HCl was added, or 4 ml of 2 N HCl was added as a blank control. The mixture was incubated for 1 hr at room temperature. The protein was precipitated with an equal volume of 20% trichloroacetic acid and was washed three times with ethanol/ethyl acetate (1:1 v/v). The final precipitate was dissolved in 2 ml of 6 M guanidine hydrochloride (pH 2.3), and insoluble debris was removed by centrifugation. The absorbance of the DNPH derivatives was measured at 360 nm. The concentration of carbonyl groups was calculated by using an absorbance coefficient 22 nM/cm and expressed as nanomole carbonyl per milligram of protein.

Isolation of Mitochondria. The hearts weighing 70–100 mg were rapidly removed and rinsed in saline. The tissue was blot-dried with filter paper and quickly transferred to ice-cold buffer A (220 nm D-mannitol, 70 mM sucrose, 10 mM HEPES, 0.1 mM EDTA, 0.5 mM EDTA, 1 mM PMSF, pH 7.4). The heart tissue was minced and gently homogenized (12 strokes) in a Teflon homogenizer. The homogenate was centrifuged at 1000 *g* for 10 mins to remove debris. The supernatant was collected in a clean tube and recentrifuged at 10,000 *g* for 15 mins. The resultant supernatant was saved as the cytosolic fraction, whereas the pellet was resuspended in 1 volume buffer A and centrifuged at 10,000 *g* for 15 mins. The supernatant was discarded and the pellet was resuspended in 1 volume of buffer B (220 mM D-mannitol, 70 mM sucrose, 10 mM HEPES, 0.1 mM EDTA, 1 mM PMSF, pH 7.4) and centrifuged at 10,000 *g* for 15 mins. The resulting brown pellet was the mitochondrial fraction.

Total GSH Concentration in Cardiac Mitochondria and Cytosol. The mitochondrial pellet was centrifuged at 10,000 *g* for 5 mins after mixing with nine volumes of 5% 5-sulfosalicylic acid (SSA). The cytosolic fraction was mixed with 20% SSA (3:1) and centrifuged at 10,000 *g* for 5 mins. The supernatants were assayed for GSH by the 5,5′-dithiobis (2-nitrobenzoic acid; DTNB)-glutathione reductase recycling assay. The total of 1.0 ml reaction mixture contained 190 µl of stock buffer (143 mM sodium phosphate and 6.3 mM Na-EDTA, pH 7.5), 700 µl of 0.248 mg of NADPH/ml of stock buffer, 100 µl of 6 mM DTNB, and 10 µl of sample. The assay was initiated by the addition of 10 µl of 266 U/ml glutathione reductase. The absorbance was measured at 412 nm for 4 mins every 20 secs (25°C). GSH was used as a standard and was assayed in parallel under the same condition as the tissue samples. The total GSH concentration was expressed as nanomoles of GSH per milligram of protein.

Statistical Analyses. Data are expressed as mean ± SD and were analyzed according to a 2 × 2 (ethanol vs. antioxidant) factorial design. After a significant interaction was detected by the two-way analysis of variance (ANOVA), the significance of the main effects was further determined. The level of significance was considered at $P < 0.05$.

Results

Blood Alcohol Level. At 6 hrs after ethanol administration by gavage, the BALs in the ethanol-treated mice were measured in comparison with controls, and the effect of NAC pretreatment on the rise in BALs was also determined, as shown in Figure 1. A significant rise in the BALs 6 hrs after ethanol administration was observed, and pretreatment with NAC did not affect the elevation of BALs.

Alcohol-Induced Myocardial Injury. Acute alcohol administration did not change the weight of the heart as estimated by the ratio of heart weight to body weight. NAC pretreatment neither caused heart weight change nor altered the effect of alcohol administration (data not shown). The total protein levels in the heart as estimated by the amount of protein content per gram of heart tissue were not changed by either alcohol or NAC or their combination (data not shown). To estimate the overall heart damage by acute alcohol exposure and the effect of NAC pretreatment, serum CPK activity was measured 6 hrs after ethanol administration. As shown in Figure 1, acute ethanol administration caused a significant increase in the serum CPK activity, and this elevation was significantly suppressed by NAC pretreatment. The increase in the serum CPK activity thus indicated myocardial damage. Therefore, ultrastructural changes of myocardial tissue induced by acute alcohol administration were determined by an electron microscopic method. The result presented in Figure 2 shows that acute alcohol administration caused myocardial ultrastructural damage. In particular, mitochondrial structural abnormalities were observed, including mitochondrial swelling, variations in the size and shape, disarray, and reduction in

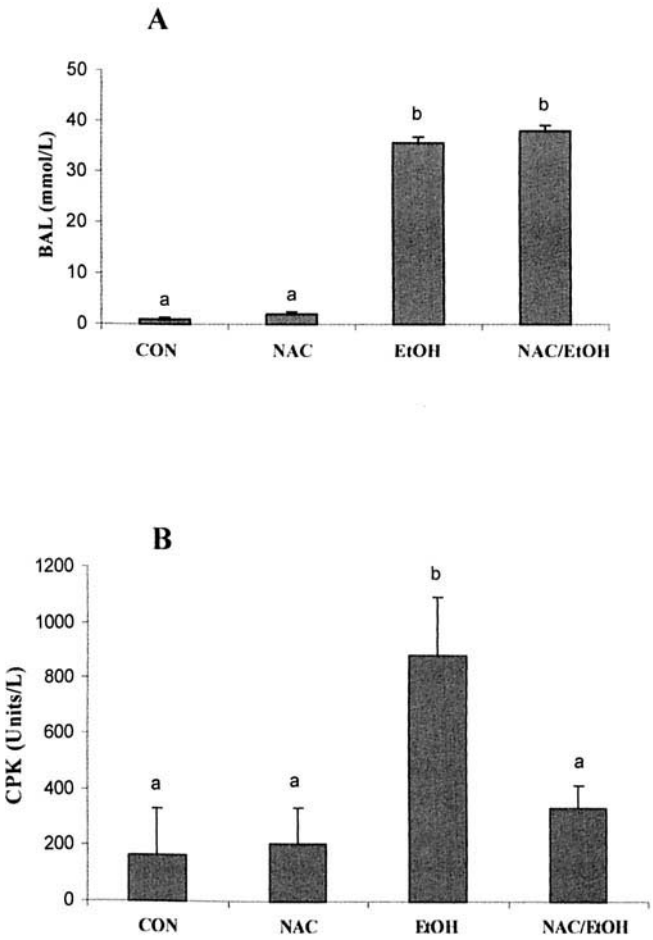

Figure 1. Blood alcohol levels (BAL) and serum creatine phosphokinase (CK) activities in the acute ethanol-treated mice and the effect of NAC pretreatment. Data are mean ± SD values ($n = 3$–9). Groups that do not share the same letter are significantly different from each other ($P < 0.05$).

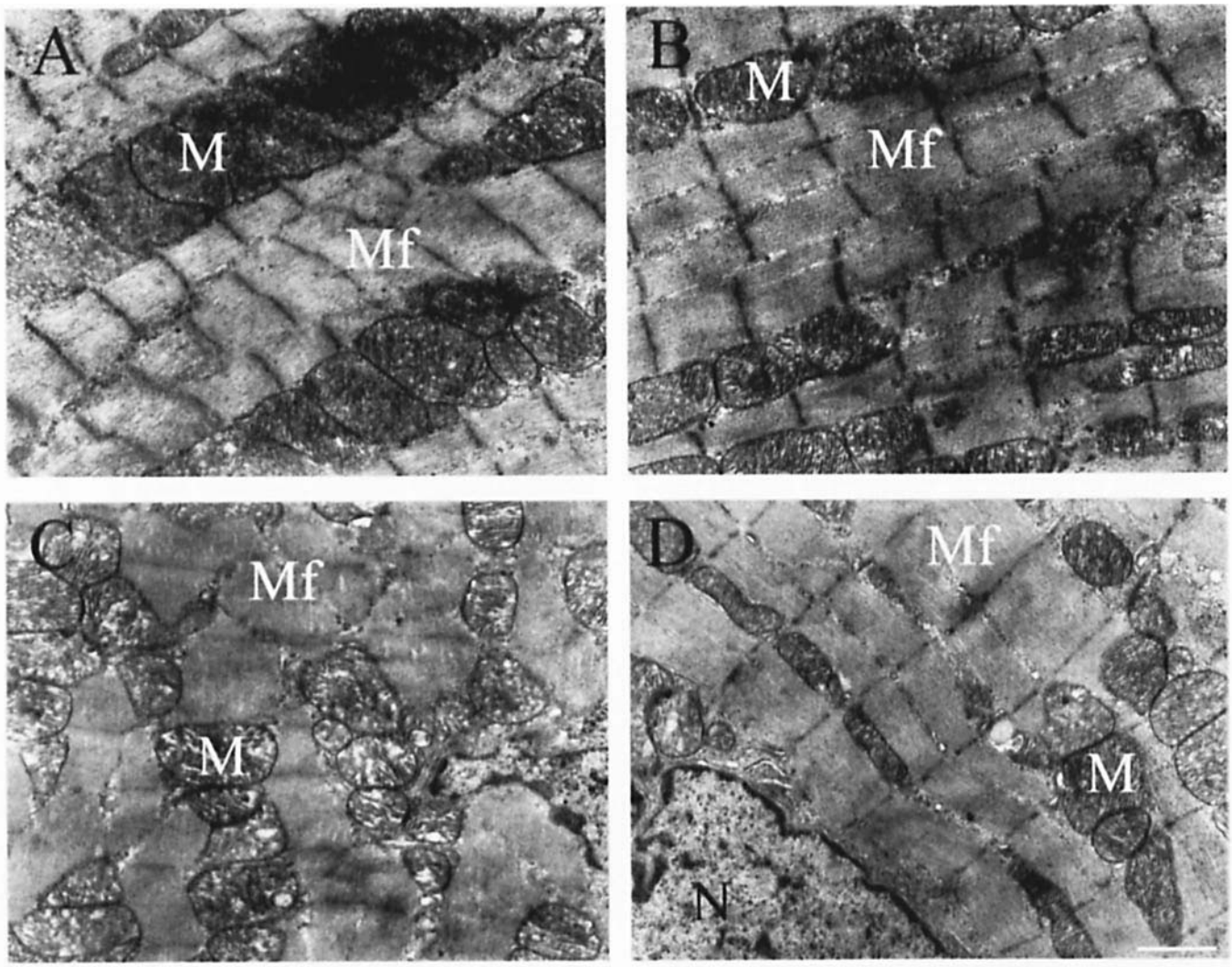

Figure 2. Acute ethanol-induced ultrastructural changes in the cardiomyocyte (C) and the effect of NAC pretreatment (D). Ethanol-induced ultrastructural damage mostly involved mitochondrial (M) structural changes, including mitochondrial swelling, disarray, and reduction in the number of cristae, in comparison with control (A). NAC pretreatment alone (B) did not cause changes in the ultrastructures but inhibited alcohol-induced damage. Scale bar = 1 μm.

the number of cristae. These alcohol-induced changes were all inhibited in the NAC pretreated mice.

Alcohol-Induced Myocardial Oxidative Stress. An important method to define oxidative stress is the measurement of ROS-induced macromolecular damage such as lipid peroxidation as measured by the thiobarbituric acid reactive substances (TBARS) concentrations in tissue extract and protein oxidation through the measurement of protein carbonyl concentrations. The TBARS assay was used to detect the concentration of malondialdehyde derivatives in the heart tissue. The result shown in Figure 3 demonstrates that acute ethanol administration significantly elevated lipid peroxide levels and NAC significantly inhibited alcohol-induced lipid peroxidation. Alcohol also significantly increased the protein carbonyl concentrations in the heart, which was also significantly inhibited by NAC (Fig. 3).

Alcohol-Induced Myocardial Mitochondrial GSH Depression. To determine the effect of acute alcohol administration on myocardial antioxidant activity, we measured GSH concentrations in mitochondrial and cytosolic compartments of the heart after separating the two compartments by fractional centrifugation. The results presented in Figure 4 show that acute alcohol exposure caused a significant decrease in the mitochondrial GSH concentrations (by 36%). In the cytosolic compartment, a small (15%), but not significant, decrease was observed. The alcohol-induced GSH decreases in both compartments were significantly inhibited by NAC pretreatment, although NAC alone did not elevate GSH concentrations in either compartment.

Discussion

The data obtained from this study demonstrate that acute ethanol administration in mice caused significant

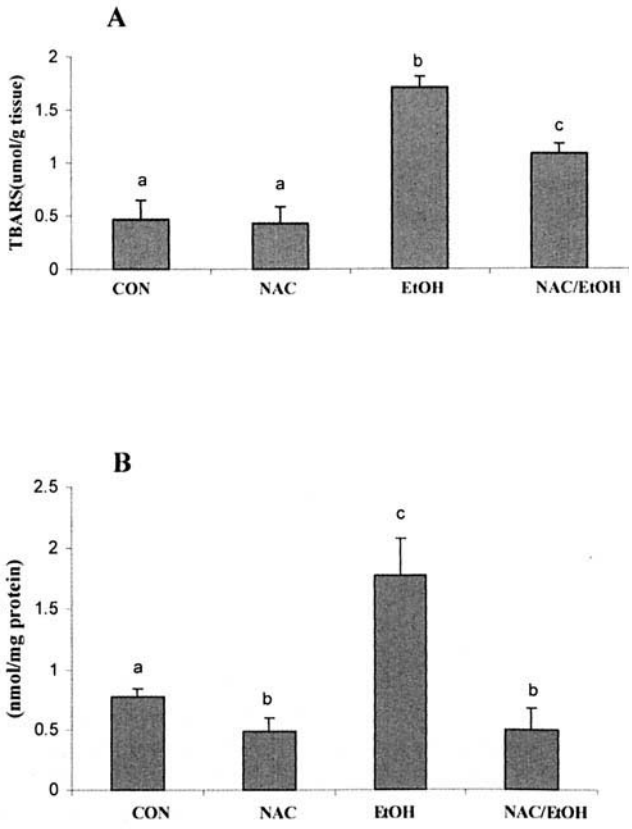

Figure 3. The effect of NAC pretreatment on (A) acute alcohol-induced cardiac lipid peroxidation (TBARS content) and (B) protein carbonyl levels. Data are mean ± SD values (n = 6–9). Groups that do not share the same letter are significantly different from each other ($P < 0.05$).

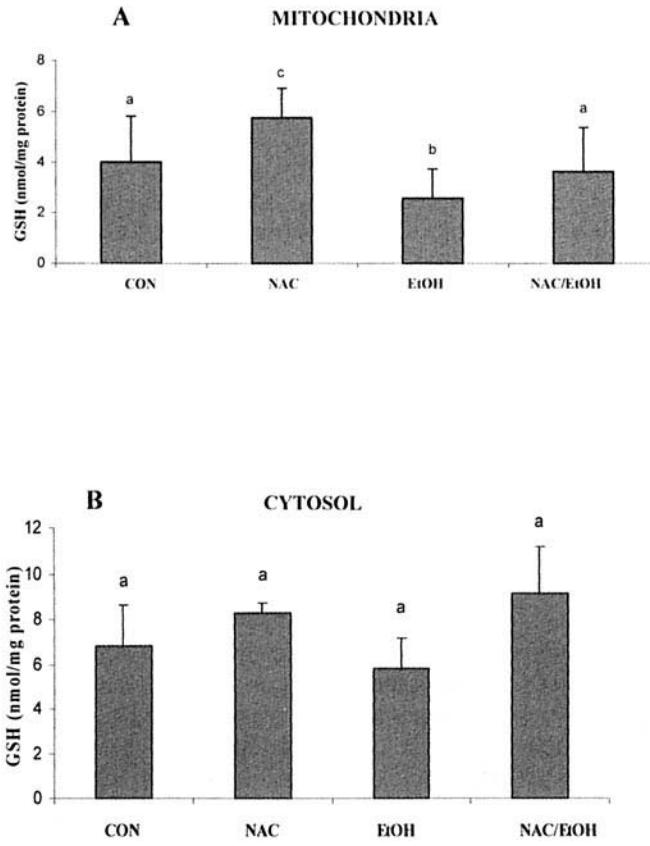

Figure 4. Changes in cardiac mitochondrial and cytosol GSH concentrations induced by acute ethanol administration and the effect of NAC pretreatment. Data are mean ± SD values (n = 15). Groups that do not share the same letter are significantly different from each other ($P < 0.05$).

cardiac injury, which was revealed by the elevation of serum CPK activities and the ultrastructural alterations in the cardiac tissue. In association with the cardiac tissue injury, acute ethanol administration induced significant increases in lipid peroxidation and protein oxidation and marked decreases in the cardiac mitochondrial GSH concentrations. These oxidative damages to lipids and proteins along with the decrease in endogenous antioxidant capacity due to the reduced GSH concentrations indicate alcohol-induced oxidative stress. The link between the oxidative stress and myocardial injury was established by the observation that supplementation with NAC, a well-known antioxidant, significantly suppressed all of the detrimental effects of acute alcohol exposure.

Alcoholic cardiomyopathy in humans has been recognized for a long time. Most experimental animal studies have focused on a relative long-term administration of alcohol to generate a chronic detrimental effect on the heart. Such chronic studies are important to determine the structural and functional changes under the influence of alcohol; however, the direct alcohol effect on the heart is difficult to sort out because of multiple confounding factors such as the role of alterations in the immune system and changes in the metabolism of organ systems. Several studies have shown that a single high dose of alcohol mimicking binge drinking in humans produced liver oxidative stress and injury (19, 20, 25). The results obtained from the present study clearly demonstrate that the heart also suffers the same oxidative damage as the liver from a single high-dose alcohol administration. This damage would reflect the direct detrimental action of alcohol and/or its metabolites in the heart.

The ultrastructural changes in the hearts of alcohol-treated mice under electron microscope would represent an early detrimental response of the myocardial tissue to alcohol. The defects in the mitochondrial architecture would lead to the damage of the mitochondrial metabolism, resulting in an increased level of fatty esters in the alcohol-intoxicated heart and thus become a key contributor to intrinsic cell dysfunction (26). Recent reports have shown that abnormalities in calcium homeostasis associated with the opening of the mitochondrial permeability transition pore (MPTP) may be responsible for the observed mitochondrial swelling and membrane disruption (27).

Acute alcohol administration also results in alterations of mitochondrial composition and fluidity, which would impair mitochondrial function (28).

These detrimental changes in mitochondrial structure and function would make significant contributions to the generation of ROS and oxidative stress. Under oxidative stress conditions, macromolecules such as lipids and proteins suffer from oxidative damage leading to accumulation of the oxidized products in the cell. The oxidative damages to lipids and proteins in the alcohol-treated heart were thus detected by measuring the levels of lipid peroxidation and protein oxidation. These results indicate that acute alcohol administration did cause oxidative stress in the heart, as observed in the liver.

The acute alcohol administration–induced myocardial injury in association with oxidative stress is an important finding of the present study. The critical element to define the cause-and-effect relationship between oxidative stress and cardiac injury was the use of the antioxidant NAC. NAC, through intracellular metabolism, delivers cysteine, which supports the critical substrate for GSH synthesis and which itself can also function as an ROS scavenger. The link between oxidative stress and myocardial injury by acute alcohol administration is thus established by the fact that NAC equally suppressed alcohol-induced myocardial lipid peroxidation and protein oxidation, as well as myocardial injury.

What is possibly responsible for the oxidative stress induced by acute alcohol exposure? GSH is involved in several reactions in the body and is one of the most prominent nonenzymatic antioxidants (29). Our results showed that acute ethanol administration produced a significant decrease in the mitochondrial GSH concentrations and a slight decrease in cytosolic GSH. One major reason for this greater decrease in mitochondrial GSH may relate to the fact that GSH is not synthesized within the mitochondria and the mitochondrion depends on a specific GSH transporter to obtain GSH from cytosol (30). This specific GSH transporter may be sensitive to alcohol and/or its metabolites, thus leading to its inhibition under the alcohol-intoxication conditions. In the mitochondrion, the GSH/GSH peroxidase system plays the most prominent role in the defense from oxidative injury because of the lack of catalase in this important organelle (31). The decrease in GSH concentrations in the mitochondria would thus be highly responsible for ROS generation and the structural and functional damage in this organelle.

The effect of acute alcohol administration on mitochondrial structure and function has also been observed in previous studies. In a rat model, acute alcohol exposure led to depressed mitochondrial respiration in the heart (32). The same ultrastructural changes have also been observed in cultured cardiomyocytes exposed to ethanol (33). Interestingly, it has been shown that acute ethanol exposure reduced the rate of protein synthesis in the rat heart (34, 35). However, we have not observed any decrease in the total protein content in the heart of mice exposed to a high dose of alcohol for 6 hrs. Determining whether this discrepancy is the result of species difference would be an interesting investigation for future studies. Another interesting aspect is that we observed that acute alcohol administration significantly elevated protein carbonyl concentrations in the heart. However, a study performed by Reilly *et al.* (36) has shown that acute ethanol exposure did not increase protein carbonyl content in rat skeletal muscle. Together, these observations raise a question as to whether there are different responses to acute alcohol exposure between cardiac and skeletal muscles, or whether there is simply a species difference.

Although this study clearly demonstrates the link between oxidative stress and myocardial injury induced by acute alcohol exposure, there are several limitations. First, the oxidative injury induced by acute alcohol exposure may not be applied to chronic alcohol exposure. It is possible that oxidative stress is also involved in the pathogenesis of chronic alcohol-induced cardiac injury; however, the mechanisms of oxidative stress may not be the same between acute and chronic exposures. In chronic alcohol exposure, many factors such as pro-inflammatory cytokines, immune responses, and altered metabolisms are all possibly involved in the constitution of oxidative stress conditions. Second, the results presented in this study did not indicate whether the myocardial injury is reversible. We speculate that these damages would be repairable; however, if the same acute injury occurs repeatedly, a cumulative damage to the heart would make it irreversible. Third, we demonstrated the occurrence of oxidative stress; however, the mechanism of ROS and reactive nitrogen species (RNS) generation by alcohol and its metabolites and their differential contributions to the overall oxidative injury have not been probed, which would be an important undertaking in the future.

In conclusion, this study showed that acute alcohol administration to mice caused significant cardiac damage in association with oxidative stress. In particular, alcohol-induced myocardial mitochondrial alteration was the most prominent subcellular event. Although both cytosolic and mitochondrial GSH were affected by acute alcohol exposure, only the mitochondrial GSH was significantly decreased. The selective depletion of mitochondrial GSH may be partially responsible for the observed mitochondrial damage, which in turn would be responsible for oxidative stress. The result obtained from the supplementation with NAC showed that the antioxidant significantly inhibited oxidative stress and cardiac injury induced by acute alcohol exposure. Thus, the use of antioxidants as a potential therapeutic agent in alcohol abusers should be further explored.

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1. McKenna CJ, Codd MB, McCann HA, Sugrue DD. Alcohol consumption and idiopathic dilated cardiomyopathy: a case control study. Am Heart J 135:833–837, 1995.
2. Manolio TA, Levy D, Garrison RJ, Castelli WP, Kannel WB. Relation of alcohol intake to left ventricular mass: the Framingham study. J Am Col Cardiol 17:717–721, 1991.
3. Piano RM. Alcohol and heart failure. J Card Fail 8(4):239–246, 2002.
4. Thomas AP, Sas EJ, Tun-Kirchmann TT, Rubin E. Ethanol inhibits electrically induced calcium transients in isolated rat cardiomyocytes. J Mol Cell Cardiol 21:555–565, 1989.
5. Nordmann R, Ribiere C, Rouach H. Implication of free radical mechanisms in ethanol-induced cellular injury. Free Radical Biol Med 12:219–240, 1992.
6. 6. Klatsky AL, Friedman GD, Siegelaub AB, Gerard MJ. Alcohol consumption and blood pressure. N Eng J Med 296:1194–1200, 1977.
7. 7. Byrne-Quinn E, Fessas C. Beriberi heart disease in London. Br Med J 4:8–25, 1969.
8. Kupari M, Koskinen P, Suokas A. Left ventricular size, mass and function in relation to the duration and quantity of heavy drinking in alcoholics. Am J Cardiol 67:274–279, 1991.
9. Fernandez-Sola J, Estruch R, Nicolas JM, Pare JC, Sacarrella E, Antunrez E, Marquez AU. Comparison of alcoholic cardiomyopathy in women versus men. Am J Cardiol 80:481–485, 1997.
10. Ribiere C, Hininger I, Rouach H, Nordmann R. Effects of chronic ethanol administration on free radical defense in rat myocardium. Biochem Pharmacol 44:1495–1500, 1992.
11. Heaton MB, Paiva M, Mayer J, Miller R. Ethanol-mediated generation of reactive oxygen species in developing rat cerebellum. Neurosci Lett 334(2):83–86, 2002.
12. Reinke LA, Lai EK, DuBose CM, McCay PB. Reactive free radical generation *in vivo* in heart and liver of ethanol-fed rats: correlation with radical formation *in vitro*. Proc Natl Acad Sci U S A 84:9223–9227, 1987.
13. Esktrom G, Ingelman-Sundberg M. Rat liver microsomal NADPH-supported oxidase activity and lipid peroxidation dependent on ethanol-inducible cytochrome P450. Biochem Pharmacol 38:1313–1319, 1989.
14. Amici A, Levine RL, Tsai L. Conversion of amino acid residues in proteins and amino acid homopolymers to carbonyl derivatives by metal catalyzed oxidative reactions. J Biol Chem 264:3341–3346, 1989.
15. Paradis V, Kollinger M, Fabre M, Holstege A, Poynard T, Bedossa P. *In situ* detection of lipid peroxidation by products in chronic liver diseases. Hepatology 26:135–142, 1997.
16. 16.Reed DJ, Fariss MW. Glutathione depletion and susceptibility. Pharmacol Rev 36:S25–S33, 1984.
17. 17.Hirano T, Kaplowitz N, Tsukamoto H, Kamimura S, Fernandez-Checa JC. Hepatic mitochondrial glutathione and progression of experimental alcoholic liver disease in rats. Hepatology 16:1423–1427, 1992.
18. Chen Y, Saari JT, Kang YJ. Weak antioxidant defenses make the heart a target for damage in copper-deficient rats. Free Radical Biol Med 17:529–536, 1994.
19. Zhou Z, Sun X, Kang YJ. Metallothionein protection against alcoholic liver injury through inhibition of oxidative Stress. Exp Biol Med 227:214–222, 2002.
20. Zhou Z, Wang L, Song Z, Lambert JC, McClain CJ, Kang YJ. A critical involvement of oxidative stress in acute alcohol-induced hepatic TNF-α production. Am J Pathol 163:1137–1146, 2003.
21. Carson EJ, Pruett SB. Development and characterization of a binge drinking model in mice for evaluation of the immunological effects of ethanol. Alcohol Clin Exp Res 100:1501–1506, 1997.
22. Kang YJ, Chen Y, Yu A, Voss-McCowan M, Epstein PN. Over-expression of metallothionein in the heart of transgenic mice suppresses doxorubicin cardiotoxicity. J Clin Invest 100:1501–1506, 1997.
23. Sun X, Zhou Z, Kang YJ. Attenuation of doxorubicin chronic toxicity in metallothionein-overexpressing transgenic mouse heart. Cancer Res 61:3382–3387, 2001.
24. Oliver IT. A spectrophotometric method for the determination of creatine phosphokinase and myokinase. Biochem J 61:116–121, 1995.
25. Zhou Z, Sun X, Kang YJ. Ethanol-induced apoptosis in mouse liver: Fas- and cytochrome *c*-mediated caspase-3 activation pathway. Am J Pathol 159:329–338, 2001.
26. Beckemeier ME, Bora PS. Fatty acid ethyl esters: potentially toxic products of myocardial ethanol metabolism. J Mol Cell Cardiol 30:2487–2494, 1999.
27. 27. Duchen MR. Mitochondria and Ca^{2+} in cell physiology and pathophysiology. Cell Calcium 28:339–348, 2000.
28. 28. Gianluigi V, Ignazio G, Emanuele A, Gaetano S, Piero P, Filomena P, Giuseppe P. Mitochondrial oxidative damage and myocardial fibrosis in rats chronically intoxicated with moderate doses of ethanol. Toxicol Lett 123:209–216, 2001.
29. Meister A, Anderson ME. Glutathione. Ann Rev Biochem 52:711–722, 1991.
30. Fernandez-Checa JC, Ookhtens M, Kaplowitz N. Effects of chronic ethanol feeding on glutathione on rat hepatocyte glutathione. Relationship of cytosolic glutathione to efflux and mitochondrial sequestration. J Clin Invest 83:1247–1252, 1989.
31. Zhou Z, Kang YJ. Cellular and subcellular localization of catalase in the heart of transgenic mice. J Histochem Cytochem 48:585–594, 2000.
32. Segel LD, Mason DT. Acute effects of acetaldehyde and ethanol on rat heart mitochondria. Res Commun Chem Pathol Pharmacol 25:461–474, 1979.
33. Mikami K, Sato S, Watanabe T. Acute effects of ethanol on cultured myocardial cells: an ultrastructural study. Alcohol 25:651–660, 1990.
34. Siddiq T, Salisbury JR, Richardson PJ, Preedy VR. Synthesis of ventricular mitochondrial proteins *in vivo:* effect of acute ethanol toxicity. Alcohol Clin Exp Res 17:894–899, 1993.
35. Siddiq T, Richardson PJ, Mitchell WD, Teare J, Preedy VR. Ethanol-induced inhibition of ventricular protein synthesis *in vivo* and the possible role of acetaldehyde. Cell Biochem Funct 11:45–54, 1993.
36. Reilly ME, Mantle D, Salisbury J, Peters TJ, Preedy VR. Comparative effects of acute ethanol dosage on liver and muscle protein metabolism. Biochem Pharmacol 60:1773–1785, 2000.