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Acute Ethanol Administration Oxidatively Damages and Depletes Mitochondrial DNA in Mouse Liver, Brain, Heart, and Skeletal Muscles: Protective Effects of Antioxidants

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ABSTRACT

Ethanol metabolism causes oxidative stress and lipid peroxidation not only in liver but also in extra-hepatic tissues. Ethanol administration has been shown to cause oxidative degradation and depletion of hepatic mitochondrial DNA (mtDNA) in rodents, but its in vivo effects on the mtDNA of extra-hepatic tissues have not been assessed. We studied the effects of an acute intragastric ethanol administration (5 g/kg) on brain, heart, skeletal muscle, and liver mtDNA in mice. Ethanol administration caused mtDNA depletion and replacement of its supercoiled form by linearized forms in all tissues examined. Maximal mtDNA depletion was about similar (ca. 50%) in all organs studied. It occurred 2 h after ethanol administration in heart, skeletal muscle, and liver but after 10 h in brain. This mtDNA depletion was followed by increased mtDNA synthesis. A secondary, transient increase in mtDNA levels occurred 24 h after ethanol administration in all organs. In hepatic or extra-hepatic tissues, mtDNA degradation and depletion were prevented by 4-methylpyrazole, an inhibitor of ethanol metabolism, and attenuated by vitamin E, melatonin, or coenzyme Q, three antioxidants. In conclusion, our study shows for the first time that ethanol metabolism also causes oxidative degradation of the mitochondrial genome in brain, heart, and skeletal muscles. These effects could contribute to the development of (cardio-)myopathy and brain injury in some alcoholic patients. Antioxidants prevent these effects in mice and could be useful in persevering drinkers.

Alcoholic patients can develop not only liver lesions but also pancreatitis, brain damage, peripheral neuropathy, cardiomyopathy, and skeletal muscle myopathy (Fromenty and Pessayre, 1995; Neiman, 1998). Reactive oxygen species (ROS) and free radicals are generated during ethanol metabolism, causing oxidative stress and lipid peroxidation in liver (Fromenty and Pessayre, 1995; Kurose et al., 1996), brain (Calabrese et al., 1998), heart (Nordmann et al., 1992), and skeletal muscles (Adachi et al., 2000).

In hepatocytes, ethanol-induced free radical and ROS generation involves mitochondria, microsomal cytochrome P450 2E1 (CYP2E1), and ferrous iron and to a lesser extent, peroxisomes and cytosolic xanthine and aldehyde oxidases (Kukielka and Cederbaum, 1992; Fromenty and Pessayre, 1995; Tsukamoto, 2000). Macrophagic NADPH oxidase is another important source of ROS in the liver of alcoholized animals (Kono et al., 2000).

Mitochondria are major targets for ethanol toxicity in liver, brain, heart, skeletal muscles, and exocrine pancreas (Fromenty and Pessayre, 1995; Baker and Kramer, 1999; Cahill et al., 1999). In the liver, acute and chronic ethanol intoxication causes oxidative damage to mitochondrial proteins, phospholipids (cardiolipin), and mitochondrial DNA (mtDNA) (Fromenty and Pessayre, 1995; Wieland and Lauterburg, 1995; Cahill et al., 1999; Cederbaum, 1999). Acute intragastric ethanol administration (5 g/kg) causes oxidative degradation and depletion of hepatic mtDNA in mice (Mansouri et al., 1999), and the prevalence of hepatic mtDNA deletions is increased in alcoholic patients (Fromenty et al., 1995; Mansouri et al., 1997). However, in contrast to hepatic mtDNA, there is no information on the possible in vivo effects of ethanol on the mtDNA of extra-hepatic tissues.

In the present study, we show that the oxidative stress due to ethanol metabolism also causes extensive degradation and depletion of brain, heart, and skeletal muscle mtDNA in mice. Interestingly, the extent of mtDNA depletion in these extra-hepatic tissues is about the same as in the liver, suggesting that the ethanol-induced free radical and/or ROS formation could be as high in brain and muscles as in the liver. Importantly, we also show that the ethanol-induced mtDNA alterations can be prevented by several antioxidants

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ABBREVIATIONS: ROS, reactive oxygen species; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; CYP2E1, cytochrome P4502E1; kb, kilobase.

such as vitamin E, coenzyme Q, and melatonin. These data may suggest that in persevering alcohol abusers, antioxidants could prevent ethanol-induced hepatic and extra-hepatic lesions.

Materials and Methods

Animals and Treatments. Male Crl:CD-1(ICR)BR Swiss mice (28–30 g) from Charles River (Saint-Aubin-lès-Elbeuf, France) were fed a normal diet ad libitum (A04 biscuits; UAR, Villemoisson-sur-Orge, France). Ethanol (5 g/kg of body weight) was diluted (50:50, v/v) in water and administered by gastric intubation with an appropriate mouse-feeding stainless steel needle (Popper and Sons, New Hyde Park, NY) while control mice received water. The lethality after the single binge protocol was negligible (ca. 5%) and was mostly due to false passage.

Cyanamide was administered intraperitoneally (i.p.) (15 mg/kg) 1 h before ethanol intoxication. Silymarin (800 mg/kg) was administered i.p. 30 min before ethanol. In experiments aimed at protecting liver, heart, or skeletal muscle mtDNAs (which were all maximally depleted 2 h after ethanol administration), a single dose of melatonin (10 mg/kg) or 4-methylpyrazole (1 mmol/kg) was administered i.p. 30 min before ethanol. In experiments aimed at protecting brain mtDNA (which was maximally depleted 10 h after ethanol administration), these doses of melatonin or 4-methylpyrazole were administered 30 min before ethanol and again 3, 6, and 9 h after ethanol administration. *S*-Adenosylmethionine (25 mg/kg i.p.) was administered three times (every 2 h) before ethanol and again with ethanol. Coenzyme Q_{10} (45 mg/kg) and vitamin E (α-tocopherol) (45 mg/kg) were administered daily by gastric intubation for 5 days before ethanol intoxication. Vitamin C (100 mg/kg by gastric intubation) and dehydroepiandrosterone sulfate (0.1 mg/ml in drinking water) were given daily for 2 weeks before ethanol intoxication. α-Lipoic acid (100 mg/kg i.p.) was administered daily for 3 weeks before alcoholization. Finally, *N*-acetyl-L-cysteine (0.3% w/v in drinking water) was given for 1 month before ethanol administration. All experiments were performed in agreement with the national guidelines for the proper use of animals in biomedical research.

Isolation of Total DNA, Slot Blot Hybridization and Southern Blot Hybridization. At various points in time after ethanol and/or water administration, fragments (200–400 mg) of liver, brain, heart, and hind limb skeletal muscles were removed and immediately used for DNA extraction with QIAGEN Genomic-tip 100/G columns (QIAGEN, Hilden, Germany), according to the manufacturer's recommendations. DNA concentration was assessed from the absorption at 260 nm.

mtDNA and nuclear DNA (nDNA) were quantified by slot blot hybridization (Mansouri et al., 1999). Total DNA (200–400 ng) was blotted onto a Hybond-N+ nylon membrane (Amersham, Les Ulis, France), and hybridized with a 8.6-kb mtDNA probe generated by long polymerase chain reaction and labeled by random priming (Multiprime DNA labeling system, Amersham) (Mansouri et al., 1999). Membranes were stripped and rehybridized with a mouse C_0t-1 nDNA probe (Invitrogen, Groningen, The Netherlands). mtDNA and nDNA were assessed by densitometry analysis of autoradiographs, and the mtDNA/nDNA ratio was determined for each DNA sample (Mansouri et al., 1999). Although the within-day coefficient of variation of the mtDNA/nDNA ratio in control animals was satisfactory (averaging 25%), the between-day variation was significantly higher, thus prompting us for each set of experiments to express the values obtained in treated animals as percentage of controls.

To study the proportions of mtDNA in its supercoiled, circular, and 16.3-kb linear forms, total DNA (1.5–5 μg) was loaded on 0.7% agarose gels without ethidium bromide (Mansouri et al., 1999). DNA was electrophoresed overnight at a voltage of 2.5 V/cm, transferred to a Hybond-N+ nylon membrane, and hybridized with the 8.6-kb mouse mtDNA probe. mtDNA forms were quantified by densitometric analysis of autoradiographs (Mansouri et al., 1999).

[^{3}H]Thymidine Incorporation into nDNA and mtDNA. Ten or 18 h after ethanol and/or water administration, groups of 9 to 15 mice received [^{3}H]thymidine (0.03 μmol/kg i.p.; 0.75 mCi/kg; Amersham) and were killed 2 h later (Mansouri et al., 1999). To obtain enough DNA, the whole liver, heart, or brain of three mice or muscle fragments from three mice were pooled, homogenized in 0.25 M sucrose, and centrifuged at 600*g* for 15 min. The nuclear pellet was used for nDNA isolation using the phenol-chloroform method (Fromenty et al., 1995), while the supernatant was centrifuged at 7700*g* to isolate mitochondria (Fromenty et al., 1990), in which mtDNA was extracted by the phenol-chloroform method. nDNA and mtDNA were counted for [^{3}H]thymidine incorporation (Mansouri et al., 1999).

Statistics. Student's *t* test for independent data was used to assess the significance of differences between means, whereas one-way analysis of variance followed by a Dunnett's or Fisher's test was employed for multiple comparisons.

Results

Time Course of mtDNA Levels after Ethanol Administration. A single dose of ethanol (5 g/kg) had a biphasic effect on mtDNA levels in mouse liver, heart, skeletal muscles, and brain (Fig. 1). A similar time course of hepatic mtDNA has been observed in a previous study (Mansouri et al., 1999). Trough mtDNA levels occurred 10 h after ethanol administration in brain but 2 h after ethanol administration in liver, heart, or skeletal muscles (Figs. 1 and 2). The lowest mtDNA levels corresponded to 46, 50, 54, and 57% of control values in liver, heart, skeletal muscles, and brain, respectively (Fig. 1). mtDNA levels returned to control values between 4 and 6 h after the alcoholic binge in liver, heart, and skeletal muscles and between 12 and 24 h in brain (Fig. 1). mtDNA levels then increased above normal 24 h after the binge and finally tended to return to normal values 48 h after ethanol administration (Fig. 1).

[^{3}H]Thymidine Incorporation into mtDNA. The swift increase in mtDNA levels observed after ethanol-induced mtDNA depletion could be due to increased de novo mtDNA

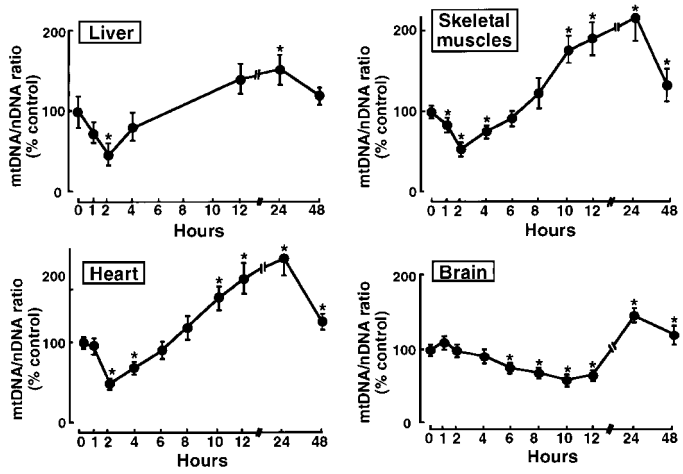

Fig. 1. mtDNA/nDNA ratio in different mouse tissues at various times after ethanol administration. Mice were killed at different points in time after intragastric ethanol (5 g/kg of b.wt.) and/or water administration. Total DNA was extracted from liver, brain, skeletal muscles, and heart, blotted on a nylon membrane, hybridized with an 8.6-kb mtDNA probe, stripped, and rehybridized with a mouse C_0t-1 nuclear DNA probe. Blot intensities were quantified by densitometry analysis. Nuclear DNA levels were not significantly modified. The mtDNA/nDNA hybridization ratio (mean ± S.E.M. for 8–16 treated mice) was expressed as the percentage of this ratio in 30 control mice. *, different from control mice, $P < 0.05$.

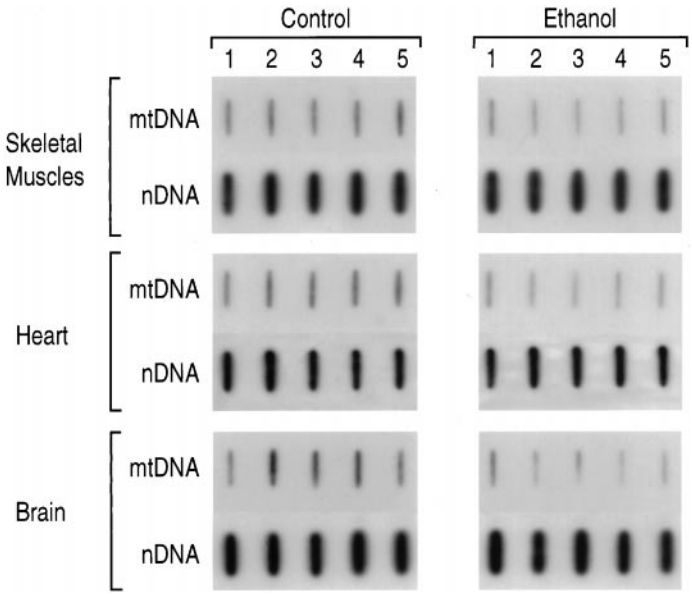

Fig. 2. Ethanol-induced mtDNA depletion in skeletal muscles, heart, and brain. Total DNA was extracted from skeletal muscles and heart 2 h after ethanol and/or water administration or from brain 10 h after the intoxication. Slot blot hybridization was performed with two probes recognizing mtDNA and nDNA, respectively. Slot blots performed with the nDNA probe ensured that the amount of deposited DNA was about similar for each mouse. A representative experiment with five control mice and five intoxicated mice is shown. Quantitative data are given in Fig. 1.

synthesis. To assess this mechanism, [^{3}H]thymidine was administered 10 or 18 h after alcohol administration, and its in vivo incorporation into DNA was studied for 2 h. [^{3}H]thymidine incorporation into mtDNA was increased 10 h after alcohol administration in liver, skeletal muscle, and heart and 18 h after ethanol administration in brain (Table 1). In contrast, [^{3}H]thymidine incorporation into nuclear DNA was either unchanged or slightly decreased (Table 1), indicating that the increased DNA synthesis only affected the mitochondrial genome.

Prevention of mtDNA Depletion by 4-Methypyrazole and Antioxidants. In a previous study, the ethanol-induced hepatic mtDNA depletion was prevented by 4-methylpyrazole, an inhibitor of ethanol metabolism, or melatonin, an antioxidant (Mansouri et al., 1999). In the present study, we tested the preventive effects of these compounds and also several others against ethanol-induced mtDNA depletion in liver, skeletal muscles, heart, and brain. Among the 11 compounds tested, only vitamin E, melatonin, 4-methylpyrazole (Fig. 3), coenzyme Q_{10} (Table 2) and α-lipoic acid (data not shown), had protective effects in the liver, whereas cyanamide, silymarin, *S*-adenosylmethionine, *N*-acetylcysteine, vitamin C, or dehydroepiandrosterone pretreatments had no effect (data not shown). Interestingly, vitamin E, melatonin, or 4-methylpyrazole also protected against ethanol-mediated mtDNA depletion in skeletal muscles, heart, and brain (Fig. 3), with 4-methylpyrazole being the most active compound in

TABLE 1

[^{3}H]Thymidine incorporation into mtDNA and nDNA after ethanol administration

[^{3}H]Thymidine was administered 10 h after ethanol and/or water administration for studies performed in liver, skeletal muscles, or heart or 18 hours after ethanol administration for studies in brain. Its incorporation into mtDNA and nDNA was studied for an additional 2 h. Results are means ± S.E.M. for the number of determinations specified in parentheses.

	Incorporation into mtDNA	Incorporation into nDNA
	cpm / μg DNA	
Liver		
Control (n = 5)	1.8 ± 0.3	3.8 ± 0.1
Ethanol (n = 5)	4.9 ± 0.2*	3.2 ± 0.3
Skeletal muscles		
Control (n = 8)	2.1 ± 0.7	3.8 ± 0.5
Ethanol (n = 8)	5.5 ± 0.8*	2.7 ± 0.2*
Heart		
Control (n = 5)	2.7 ± 0.3	3.5 ± 0.3
Ethanol (n = 6)	8.0 ± 1.3*	4.3 ± 0.4
Brain		
Control (n = 4)	0.3 ± 0.1	2.1 ± 0.2
Ethanol (n = 4)	4.4 ± 0.4*	2.1 ± 0.4

* Different from control mice, $P < 0.05$.

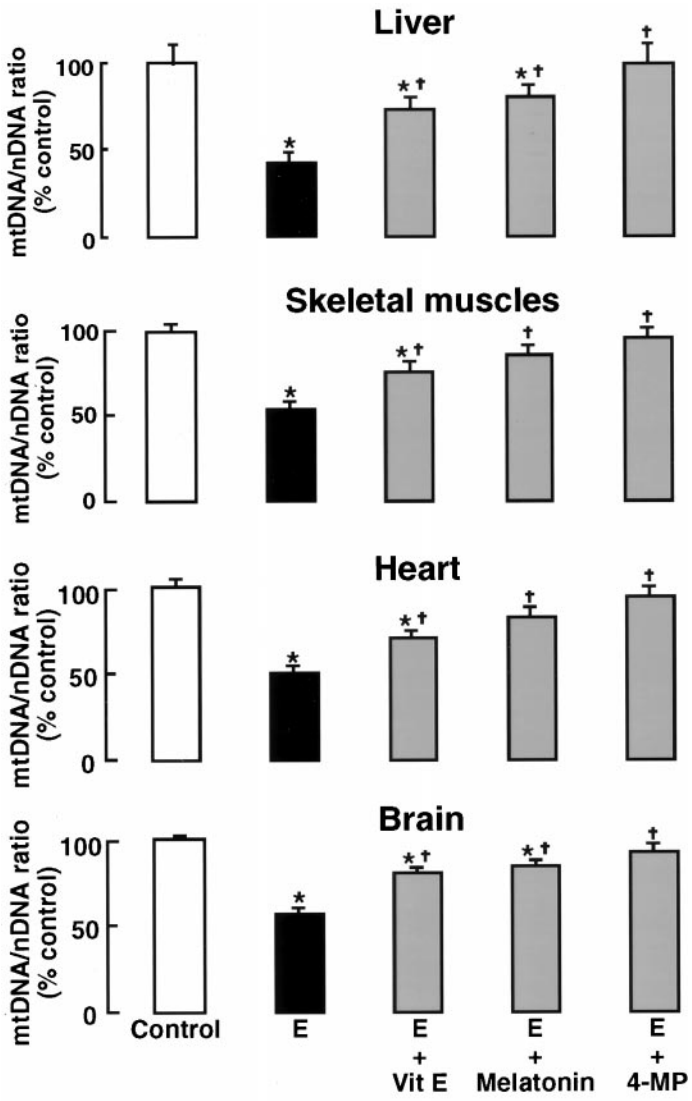

Fig. 3. Prevention of ethanol-mediated mtDNA depletion in liver, skeletal muscles, heart, and brain by vitamin E and melatonin, two antioxidants, or by 4-methylpyrazole, an inhibitor of ethanol metabolism. Mice were pretreated as described under *Materials and Methods*. The mtDNA/nDNA ratio (mean ± S.E.M. for 9–14 treated mice) was assessed by slot blot hybridization 2 h after ethanol and/or water administration in liver, skeletal muscles, and heart and after 10 h in brain. Results (mean ± S.E.M. for 10–20 treated mice) are expressed as percentages of control values (10–30 mice). E, ethanol; Vit E, vitamin E; 4-MP, 4-methylpyrazole. *Different from control mice, $P < 0.05$. †, different from unprotected ethanol-intoxicated mice, $P < 0.05$.

TABLE 2

Coenzyme Q_{10} protects against ethanol-induced mtDNA depletion and degradation in liver and heart

Water, ethanol, and coenzyme Q_{10} were administered to mice as described under *Materials and Methods*, and total DNA was extracted from liver or heart 2 h after ethanol or water administration. The mtDNA/nDNA ratio and the percentage of the three main mtDNA forms (supercoiled, circular, and linear) were assessed by slot blot and Southern blot hybridization, respectively. Results are means ± S.E.M. for five to fifteen determinations.

	mtDNA/nDNA	mtDNA		
		Supercoiled	Circular	Linear
	% control	*% mtDNA*		
Liver				
Control	100 ± 11	26 ± 9	62 ± 7	12 ± 4
Ethanol	44 ± 10*	9 ± 5	63 ± 4	28 ± 5*
Ethanol + CoQ	75 ± 6†	50 ± 9†	40 ± 4*,†	10 ± 6
Heart				
Control	100 ± 5	25 ± 8	63 ± 8	12 ± 4
Ethanol	50 ± 5*	2 ± 1*	67 ± 5	31 ± 4*
Ethanol + CoQ	83 ± 8†	67 ± 14*,†	15 ± 9*,†	18 ± 8

CoQ, coenzyme Q_{10}.
* Different from control mice, $P < 0.05$.
† Different from unprotected ethanol-intoxicated mice, $P < 0.05$.

all tissues (Fig. 3). Finally, coenzyme Q_{10} also afforded a significant, albeit partial, protection against ethanol-induced mtDNA depletion in heart (Table 2) and brain (data not shown). It was noteworthy that treatment of mice with vitamin E, melatonin, 4-methylpyrazole, or coenzyme Q did not significantly change mtDNA levels in the different tissues (data not shown), thus suggesting that their protective effect against ethanol-induced mtDNA depletion cannot be explained by an intrinsic ability to increase tissue mtDNA levels in mice.

Partial Prevention of Hepatic mtDNA Overshoot by 4-Methypyrazole. Since 4-methylpyrazole almost fully protected mice against ethanol-induced mtDNA depletion, we also assessed its ability to prevent the overshoot in mtDNA levels that followed the depletion. Mice were thus treated with either ethanol or with 4-methylpyrazole plus ethanol and were killed 24 h later for hepatic mtDNA analysis. Our results showed that whereas mtDNA levels in intoxicated mice ($n = 12$) were significantly increased by 42% compared with the controls ($n = 7$), mtDNA levels in alcoholized mice pretreated with 4-methylpyrazole ($n = 11$) were increased by only 25% (data not shown). Therefore, our data suggested that ethanol-induced mtDNA depletion cannot fully account for the overshoot of mtDNA resynthesis.

Effects on mtDNA Forms and Prevention of These Effects by 4-Methypyrazole and Antioxidants. We have shown previously that ethanol administration modifies the repartition of the different hepatic mtDNA forms as a consequence of mtDNA strand breaks (Mansouri et al., 1999). Present results indicate similar effects in skeletal muscles, heart, and brain (Figs. 4 and 5). Two hours after alcohol administration, the supercoiled form of liver, skeletal muscle, and heart mtDNA (i.e., an intact and native form) was decreased, whereas its abnormal linearized form was increased (Figs. 4 and 5). A similar pattern was observed in brain 10 h after ethanol intoxication (Fig. 5).

Present results also show for the first time that 4-methylpyrazole or melatonin (Fig. 5) and also coenzyme Q_{10} (Table 2) prevent ethanol-induced effects on hepatic mtDNA forms. Interestingly, melatonin or 4-methylpyrazole also efficiently protected mice against ethanol-induced loss of supercoiled mtDNA in skeletal muscles, heart, and brain (Figs. 4 and 5). We also verified that the coenzyme Q_{10} pretreatment also prevented ethanol-induced mtDNA strand breaks in the heart (Table 2). Finally, further experiments with mice treated with 4-methylpyrazole or melatonin alone showed that these derivatives did not increase the proportion of supercoiled mtDNA in the liver (data not shown). These results suggested that increased supercoiled mtDNA in intoxicated mice pretreated with either melatonin or 4-methylpyrazole could not be ascribed to the sole effect of these protective drugs.

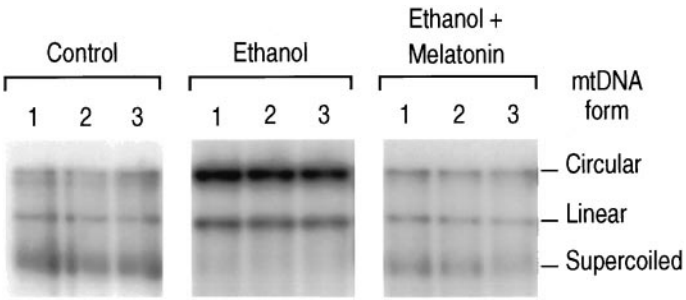

Fig. 4. Ethanol-induced changes in mtDNA forms in skeletal muscles and prevention of these changes by melatonin. Melatonin was administered as described under *Materials and Methods*, and total DNA was extracted from skeletal muscles 2 h after ethanol and/or water administration. Southern blots of total DNA (1.5 μg in control mice, 5 μg in ethanol-treated mice and, 1.5 μg in melatonin-protected, ethanol-treated mice) were hybridized with an mtDNA probe. Note the loss of supercoiled mtDNA in ethanol-intoxicated mice and the prevention of this loss in mice also treated with melatonin.

Discussion

This study shows for the first time that an alcoholic binge causes extensive mtDNA degradation and mtDNA depletion in mouse heart, skeletal muscles, and brain, followed by increased mtDNA replication and increased mtDNA levels (Figs. 1–5). These data extend a previous study showing hepatic mtDNA degradation and recovery in the same model (Mansouri et al., 1999). Importantly, the maximal serum ethanol concentrations achieved in this model are ca. 4 g/liter, a concentration that can occur in severely intoxicated patients (Mansouri et al., 1999).

In all tissues, ethanol-mediated mtDNA degradation and mtDNA depletion were fully prevented by 4-methylpyrazole, an inhibitor of both alcohol dehydrogenase and CYP2E1 (Figs. 3 and 5), suggesting that ethanol metabolism plays a major role in ethanol-mediated mtDNA damage. Ethanol

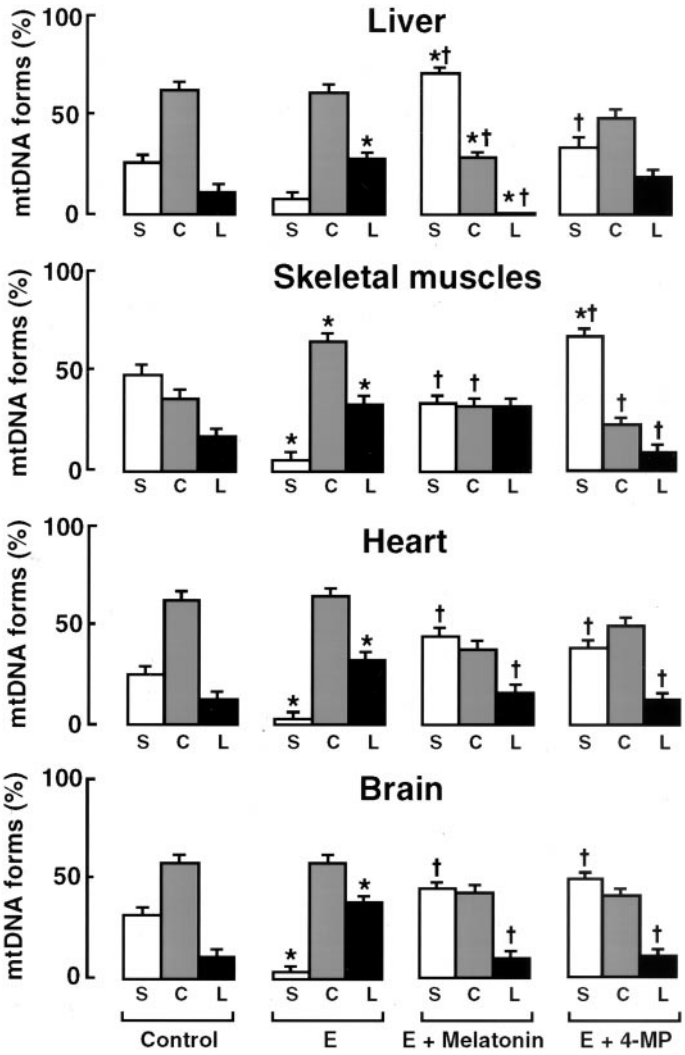

Fig. 5. Ethanol-induced changes in mtDNA forms in liver, skeletal muscles, heart, and brain and prevention of these changes by melatonin or 4-methylpyrazole. Protective substances or saline were administered as described under *Materials and Methods*. Percentages of supercoiled (S), circular (C), and full-length linear (L) mtDNA forms were determined by densitometric analysis of Southern blot autoradiographs, 2 h after intragastric ethanol and/or water administration in liver, skeletal muscles, and heart, and 10 h after ethanol administration in brain. Results are means ± S.E.M. for 8 to 18 control mice and 5 to 14 treated mice. E, ethanol; 4-MP, 4-methylpyrazole. *, different from control mice, $P < 0.05$. †, different from unprotected ethanol-intoxicated mice, $P < 0.05$.

metabolism causes acetaldehyde formation, CYP2E1-mediated 1-hydroxyethyl radical, and ROS formation, and also increases the NADH/NAD^+ ratio, which causes reduction of ferric iron to ferrous iron, a potent generator of the hydroxyl radical, which can then cause lipid peroxidation, releasing reactive aldehydes such as malondialdehyde and 4-hydroxynonenal. Acetaldehyde, ROS, the 1-hydroxyethyl radical, and lipid peroxidation products may all damage DNA (Brooks, 1997; Mansouri et al., 1999). Cyanamide, an inhibitor of acetaldehyde metabolism (Efthivoulou and Berry, 1997), was not protective in our study. Although a failure of our pretreatment protocol to confer significant protection to mice is conceivable, this result may also be due to an antagonistic effect of cyanamide, which increases levels of acetaldehyde (that may damage mtDNA) while decreasing levels of NADH (that may decrease ROS production). Clearly, further investigations are needed to determine the molecular species that trigger mtDNA depletion after an alcoholic binge.

In all tissues, ethanol-mediated mtDNA depletion was mostly prevented by vitamin E and melatonin (Figs. 3–5). These powerful antioxidants would not be expected to protect against acetaldehyde toxicity, but they can act on both ROS and ROS-mediated lipid peroxidation. Collectively, these results suggest that the increased ROS formation triggered by ethanol metabolism plays a major role in ethanol-induced mtDNA depletion. As the extent of ethanol-induced mtDNA depletion was essentially similar in liver, heart, skeletal muscles, and brain (Fig. 1), the overall formation of these mtDNA-damaging agents may be about similar in these different tissues. Because extra-hepatic tissues contain low levels of alcohol dehydrogenase and microsomal CYP2E1 (Kerr et al., 1989; Thum and Borlak, 2000; Voirol et al., 2000), other cellular components, such as mitochondria, may be mainly involved in ROS generation during ethanol intoxication. CYP2E1 is highly expressed in brain mitochondria (Bhagwat et al., 1995) and could contribute, in part, to ethanol-induced ROS formation in these organelles.

In organs with large amounts of lipids, such as the brain, ROS could oxidize unsaturated lipids causing lipid peroxidation, which in turn releases 4-hydroxynonenal and malondialdehyde, both of which react with respiratory chain polypeptides (Chen et al., 2000), thus blocking the flow of electrons along the respiratory chain, which may increase mitochondrial ROS formation (Pessayre et al., 2000). This secondary cause of sustained ROS generation might explain the more prolonged mtDNA depletion in brain rather than in organs with lower amounts of lipids, such as liver, heart, or skeletal muscles.

In the present study, mtDNA depletion in the diverse tissues was associated with major changes in the repartition of mtDNA forms. The normal, supercoiled form of mtDNA had almost disappeared, with a corresponding increase in linearized mtDNA forms (Figs. 4 and 5), showing that ethanol intoxication causes mtDNA strand breaks. Ethanol intoxication also causes the formation of oxidized DNA bases and abasic (apyrimidinic/apurinic) sites in hepatic DNA (Wieland and Lauterburg, 1995; Mansouri et al., 1999). The DNA repair machinery is incomplete in the mitochondria, and mtDNA molecules harboring too many lesions are destroyed by mitochondrial nucleases instead of being repaired (Croteau et al., 1999; Mansouri et al., 1999), thus causing transient mtDNA depletion (Mansouri et al., 1999).

As mtDNA levels are tightly regulated (Tang et al., 2000), this mtDNA depletion triggers an adaptive increase in mtDNA synthesis (Mansouri et al., 1999; Holt et al., 2000). [^{3}H]Thymidine incorporation into mtDNA is increased in liver and extra-hepatic organs (Table 1) causing prompt restoration of mtDNA levels and then increased mtDNA levels at 24 h (Fig. 1). It is noteworthy that mtDNA first tended to decrease and then significantly increase in cardiac myocytes cultured with 200 mM ethanol (Kennedy, 1997). However, our data point to additional mechanism(s) involved in mtDNA resynthesis, since 4-methylpyrazole only partially prevented the late mtDNA overshoot while almost fully protecting against ethanol-induced mtDNA depletion. Interestingly, recent data suggest that mtDNA levels can increase in response to endogenous or exogenous oxidative stress (Lee et al., 2000).

We have found multiple mtDNA deletions, all flanked by tandem repeats, in the liver of alcoholic patients (Fromenty et al., 1995; Mansouri et al., 1997). Unrepaired mtDNA damages (strand breaks, oxidized bases, and abasic sites) may be involved in the generation of such deletions (Berneburg et al., 1999; Mansouri et al., 1999). Present results showing extensive mtDNA damage in brain, heart, and skeletal muscles suggest that mtDNA deletions can also arise in the extra-hepatic tissues of alcoholic patients. Indeed, an mtDNA deletion has been demonstrated in the white blood cells of these patients (Tsuchishima et al., 2000). Alcoholic patients develop not only liver lesions but also brain damage, cardiomyopathy, and skeletal muscle myopathy (Fromenty and Pessayre, 1995; Neiman, 1998). As mitochondrial dysfunction leads to cell dysfunction or cell death (Kroemer and Reed, 2000), ethanol-induced oxidative damage to mitochondrial proteins, lipids, and DNA may play an important role in the pathogenesis of these extra-hepatic alcoholic lesions.

Although prevention of alcohol-induced injuries would best be achieved by abstinence from alcohol, some alcohol abusers keep drinking despite all recommendations and warnings, suggesting a need for preventive treatments. Among the various antioxidants tested in the present study, vitamin C, *S*-adenosylmethionine, *N*-acetylcysteine, silymarin, and dehydroepiandrosterone pretreatments were ineffective, possibly due to low lipid solubility and iron-mediated toxicity (for vitamin C) and/or relatively weak antioxidant activities (for the other compounds). In contrast, coenzyme Q_{10} (Table 2), vitamin E and melatonin (Fig. 3), or α-lipoic acid (data not shown), all exerted protective effects against ethanol-mediated hepatic mtDNA depletion. These antioxidants have lipophilic moieties that may incorporate into mitochondrial membranes. Vitamin E is present in rat liver mitochondria where it plays an important role against ROS-induced respiratory dysfunction (Bindoli, 1988). Melatonin seems to be able to bind to complex I of the respiratory chain that is embedded in the inner mitochondrial membrane (Absi et al., 2000). Coenzyme Q is a key component of the mitochondrial respiratory chain (Matthews et al., 1998), while α-lipoic acid acts as a coenzyme for mitochondrial pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (Hagen et al., 1999). Interestingly, α-lipoic acid, vitamin E, and coenzyme Q_{10} are being tried in the treatment of inborn or drug-induced mitochondrial cytopathies (Antozzi et al., 1997; Garcia de la Asuncion et al., 1998), and melatonin also prevents brain lipid peroxidation in ethanol-treated rats (El-Sokkary et al., 1999). In the present study, vitamin E or melatonin efficiently prevented ethanol-mediated mtDNA depletion not only in mouse liver, but also in brain, heart, or skeletal muscles (Fig. 3). These two antioxidants are already used in humans in other indications and could be tried in alcohol abusers who are unable or unwilling to stop drinking.

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