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Biochemical Pharmacology 64 (2002) 369–374

Biochemical Pharmacology

Rapid communication

Uncoupling protein 2 (UCP2) lowers alcohol sensitivity and pain threshold

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Accepted 3 May 2002

Abstract

Abuse of ethanol is a major risk factor in medicine, in part because of its widespread effect on the activity of the central nervous system, including behavior, pain, and temperature sensation. Uncoupling protein 2 (UCP2) is a mitochondrial protonophore that regulates cellular energy homeostasis. Its expression in mitochondria of axons and axon terminals of basal forebrain areas suggests that UCP2 may be involved in the regulation of complex neuronal responses to ethanol. We employed a paradigm in which acute exposure to ethanol induces tolerance and altered pain and temperature sensation. In *UCP2* overexpressing mice, sensitivity to ethanol was decreased compared to that of wild-type animals, while *UCP2* knockouts had increased ethanol sensitivity. In addition, *UCP2* expression was inversely correlated with the impairment of pain and temperature sensation induced by ethanol. Taken together, these results indicate that UCP2, a mitochondrial uncoupling protein previously associated with peripheral energy expenditure, is involved in the mediation of acute ethanol exposure on the central nervous system. Enhancement of UCP2 activation after acute alcohol consumption might decrease the time of recovery from intoxication, whereas UCP2 inhibition might decrease the tolerance to ethanol.

Keywords: Uncoupling proteins; Ethanol tolerance; Nociception; Transgenic animals; Knockout animals; Central nervous system

1. Introduction

Acute ethanol intake represents a socioeconomic problem [1]. Despite the billions of dollars spent on ethanol-related acute medical emergencies, 100,000 Americans are killed annually from acute ethanol exposure [2]. Abuse is a major risk factor, in part because of its widespread effect on the activity of the central nervous system, including behavior, pain, and temperature sensation [3]. Brain sites associated with ethanol-related mechanisms overlap those areas where the mitochondrial protonophore, UCP2, is expressed. UCP2 is a mitochondrial inner membrane protein that regulates cellular energy homeostasis [4], and it is expressed in mitochondria of axons and axon terminals of basal forebrain areas, including the amygdala, nucleus accumbens, and locus coeruleus [5,6]. UCP2 mRNA expression has also been found in the spinal cord [7], and UCP2 protein has been detected in the spinotrigeminal tract [6], a pathway that conveys pain and temperature sensation from the face. Because it has been suggested that UCP2 participates in the moderation of neurotransmission in the central nervous system [5] and because ethanol induces mitochondrial dysfunction [8], we explored the influence of UCP2 on ethanol sensitivity of locomotor activity and pain. We tested this hypothesis by using *UCP2* overexpressing and *UCP2* gene-trap knockout mice in an acute ethanol tolerance paradigm.

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Abbreviations: UCP2, uncoupling protein 2; UCP3, uncoupling protein 3; RCR, respiratory control ratio; and RR, righting reflex.

0006-2952/02/$ – see front matter
PII: S0006-2952(02)01167-X

2. Materials and methods

2.1. Animals

Eight-week-old female, alcohol-naïve *UCP2* overexpressing mice, maintained on a C57BL6/6J background (N = 10, mean weight 20.1 ± 0.98 g) and their wild-type littermates (N = 10, mean weight 21.16 ± 1.7 g) and 12-week-old male, alcohol-naïve *UCP2* knockout mice, maintained on a CF-1 background (N = 10, mean weight 29.77 ± 1.78 g) and their wild-type littermates (N = 10, mean weight 29.52 ± 1.36 g) were housed in a quiet environment under controlled temperature and lighting conditions (lights on 7:00 a.m. to 7:00 p.m.), with water and food freely available. Animals maintained their body weight throughout the experiment. All experimental procedures were conducted in accordance with approved institutional animal care protocols from Yale and Szeged Universities. For all experiments, we used mice of N6 or higher backcross generation and their wild-type littermates. For the *UCP2* overexpressing mice, heterozygous animals were used, while for the gene-trap knockout group, homozygous knockouts were used.

2.2. hUCP2 and hUCP3 expressing transgenic animals

Transgenic animals were produced using an 80-kb bacterial artificial chromosome containing the human *UCP2* and *UCP3* genes to construct an overexpressing transgenic mouse line. The transgene comprised the *UCP2* and *UCP3* genes, which are adjacent on chromosome 11, with their native promoters and *cis*-acting elements. Detailed descriptions of the production of these animals and their phenotypic characterization can be found elsewhere [9].

2.3. UCP2 gene-trap (knockout) mice

This gene-trap knockout mouse line was generated based on the methodology developed by Friedrich and Soriano [10]. Constructs lacking a promoter and including a reporter gene encoding a fusion protein with both β-galactosidase and neomycin phosphotransferase activity, were designed so that the expression of the reporter gene depends on its insertion within an active transcription unit. Introduction of the promoter trap constructs into embryonic stem (ES) cells by retroviral infection has led to the derivation of transgenic lines that show a variety of β-galactosidase expression patterns. This method led to a coincidental generation of a *UCP2* knockout line.

2.4. Northern and western blot analyses of gene-trap knockout mice

Cytoplasmic lysate from a normal mouse spleen was also prepared to see if there was an enrichment of UCP2 in the mitochondria. Western blot analysis was performed, using four different UCP2 antibodies, all commercially available (see below). Northern blot analyses of spleen RNA were carried out according to published protocols and probes [5] to determine the lack of UCP2 mRNA. Blots were probed with a 200-bp fragment from the 3′ untranslated region of mUCP2 as well as primers for cyclophilin as control.

2.5. Brain mitochondria preparation and uncoupling activity measurements

To test whether homozygous gene-trap mice have decreased mitochondrial uncoupling activity, we measured the respiratory control ratio of brain homogenates of homozygous and wild-type mice. Mitochondria were isolated from the hypothalamus and analyzed according to protocols published previously [5]. State 4 and 3 respiration and RCR were calculated.

2.6. Initial ethanol sensitivity

After an i.p. injection of 3.5 g ethanol/kg body weight of a filter-sterilized solution containing 20% (v/v) ethanol, in a volume of 0.022 mL/g body weight, mice were placed on their backs in a plastic U-shaped trough until they lost their RR. To determine loss of RR, each mouse was placed on its back once every 30 sec after the alcohol injection until it was unable to right itself within a 30-sec period. After the loss of RR, the number of minutes it took for a mouse to right itself on all four legs, three times in 30 sec, was recorded as latency to regain RR. The latency from the loss of RR until the ability to leave the plastic trough was regained was also recorded. The groups were compared on time of loss of RR, time to regain RR, and locomotor activity.

2.7. Acute ethanol tolerance pattern

Mice were injected (i.p.) daily for 5 consecutive days with a single dose of 3.5 g ethanol/kg body weight of a filter-sterilized solution containing 20% (v/v) ethanol, in a volume of 0.022 mL/g body weight. Alcohol-induced loss of RR, recovery of RR, and locomotor activity were measured following each administration of ethanol. Tolerance was defined as a significantly faster recovery of the RR following the consecutive alcohol injections.

2.8. Hot-plate test

Mice were placed on an enclosed metal plate heated to 55°. The time between placement of the mouse on the hot plate and the occurrence of either a hindpaw lick or a jump off the surface was recorded as the hot-plate latency. Hot-plate latency was measured before and 120 min after the ethanol injection on both day 1 and day 5 of the study. Each animal was placed on the hot plate once per measurement period. The cut-off time was 40 sec to prevent tissue injury.

2.9. Blood alcohol level measurement

Blood samples were microcentrifuged for 20 min (14,000 *g* force) at 4° and analyzed immediately. Plasma ethanol was determined by an ADH/NADH assay (Sigma Diagnostic) following the specifications of the manufacturer. Two-way, 2×2 (genotype $\times$ time) repeated-measure ANOVAs were used for statistical analysis.

2.10. Statistical analysis

Data were expressed as means $\pm$ SEM, and the Kolgomorow–Smirnoff test was used to analyze the normal distribution. Statistical analysis was performed by ANOVA. Only groups from the same genetic background were compared, and $P \leq 0.05$ was considered significant.

3. Results

3.1. Lack of UCP2 mRNA and protein expressions in UCP2 gene-trap mice

Northern blot analysis of RNA from homozygous *UCP2* gene-trap animals showed no band for UCP2 mRNA (Fig. 1A). In western blots, the N-19 antisera from

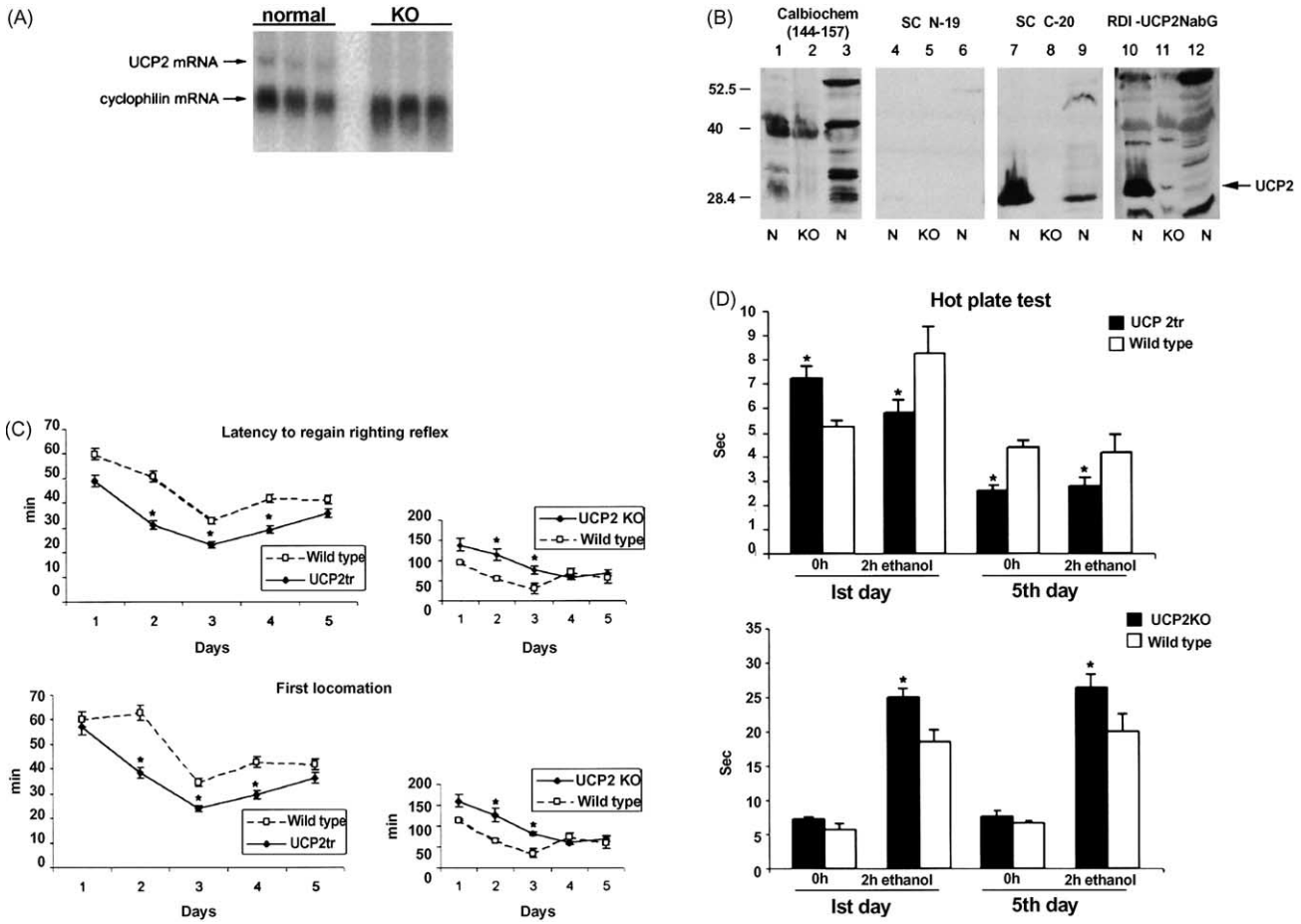

Fig. 1. (A): UCP2 mRNA expression in gene-trap knockout animals. Northern blot analyses show a lack of UCP2 mRNA expression in the spleen of *UCP2* knockout animals, while normal (wild-type) animals express this transcript. (B): UCP2 protein expression in gene-trap knockout animals. Using various commercially available antisera against UCP2, western blot analyses showed a lack of UCP2 protein expression in homozygous knockout (KO) animals, while normal (N) mice showed UCP2 protein expression. (C): Recovery from ethanol exposure of *UCP2* transgenic and gene-trap knockout animals. Human *UCP2* expressing mice (continuous line, upper left panel) showed a faster recovery from acute ethanol exposure on days 2–4 in which their regaining of righting reflex was significantly shorter than in their wild-type controls (dashed line). *UCP2* overexpressing animals (continuous line, lower left panel) started to move around sooner after ethanol exposure than wild-type littermates (dashed line). *UCP2* gene-trap knockout animals (continuous line, upper right panel) on days 1–3 needed a significantly longer time to regain their righting reflex than their wild-type controls (dashed line). *UCP2* knockout animals (continuous line, lower right panel) started to move around later than wild-type littermates (dashed line) after ethanol exposure. (D): Hot-plate test of *UCP2* transgenic and gene-trap knockout animals. During the initial testing before ethanol exposure (0 hr), *UCP2* overexpressing transgenic animals (filled columns, upper graph) moved away from the hot plate slower (∗: $P < 0.05$) than wild-type littermates (empty columns); however, after ethanol exposure (2 hr ethanol), transgenic animals moved away faster than wild-type littermates (∗: $P < 0.05$). On day 5, both before (0 hr) and after ethanol exposure (2 hr ethanol), transgenic animals moved away faster then wild-type littermates (∗: $P < 0.05$). During the initial testing before ethanol exposure (0 hr), *UCP2* knockout animals (filled columns, lower graph) moved away from the hot plate at a pace similar to that of their wild-type littermates; however, 120 min after ethanol exposure (2 hr ethanol), knockout animals moved away slower (∗: $P < 0.05$) than wild-type animals on both days 1 and 5.

Santa Cruz Biotechnology Inc. did not detect UCP2 (Fig. 1B, lanes 4–6). The antisera against 144–157 amino acid residues by Calbiochem Inc. slightly detected a diffuse protein band of around 32 kDa that was reduced in the gene-trap mice (Fig. 1B, lanes 1–3). The C-20 antisera from Santa Cruz Biotechnology Inc. strongly detected a protein of 32 kDa that was missing completely in the *UCP2* gene-trap mouse spleen (Fig. 1B, lanes 7–9). This protein was also detected in the cytoplasmic lysate (CL) from a normal mouse, but at a much lower level, supporting the location of UCP2 in the spleen mitochondria. The UCP2 antisera from Research & Development Systems Inc. also detected the 32 kDa protein, that was detected by the SC C-20 (Fig. 1B, lanes 10–12), which was missing in the gene-trap mice. This antibody, however, showed strong cross-reactivity with a number of other proteins. The high cross-reactivity of this antibody was also evident in the CL sample (Fig. 1B, lane 12). Therefore, three of the four UCP2 antibodies detected a protein in spleen mitochondria that we identify as UCP2, since it was missing in the spleens of the *UCP2* gene-trap mice.

3.2. RCR of UCP2 gene-trap knockout animals

To further test whether the *UCP2* gene-trap animals are functional *UCP2* knockout animals, we assessed the RCR of brain homogenates of gene-trap animals and their wild-type controls. The RCR is a number derived from the division of oxygen consumption at State 3 respiration (phosphorylating mitochondria) by oxygen consumption at State 4 respiration (non-phosphorylating mitochondria in which oxygen consumption is due exclusively to proton leak). Higher RCR values correspond with lower uncoupling. In support of being functional *UCP2* knockout animals, the RCR value of homozygous gene-trap mice was significantly higher than that of the wild-type controls (3.24 ± 0.1 vs. 2.59 ± 0.09; $P < 0.05$).

3.3. Initial ethanol sensitivity (Fig. 1C upper panels)

UCP2 overexpressing animals regained RR in a shorter time than the controls, but the difference was not statistically significant. *UCP2* knockout mice showed significantly higher sensitivity after the first ethanol injection when the latencies to regain RR and locomotion were compared ($P < 0.05$; Fig. 1C and D).

3.4. Acute ethanol tolerance (Fig. 1C lower panels) of UCP2 overexpressing mice

Both the transgenic and control mice recovered more rapidly following the second alcohol injection, but this was significant in only the *UCP2* overexpressing animals ($P < 0.05$). The initial sensitivity difference remained constant throughout the experiment, and it was significant on days 2, 3, and 4 of treatment ($P < 0.05$). We observed a further increase in tolerance after the third injection in both groups. On days 4 and 5 of ethanol treatment, an increased recovery time was observed in both the transgenic and control animals. The recovery of locomotion was delayed compared to the regaining of RR after the first three injections, but was the same after days 4 and 5 of ethanol administration.

3.5. UCP2 knockout mice

The initial sensitivity difference in which knockout animals regained their RR slower ($P < 0.05$) than their wild-type littermates remained in the first 3 days, but was diminished on treatment days 4 and 5. The recovery of locomotion was faster in the control mice than in the *UCP2* knockout animals ($P < 0.05$).

3.6. Hot-plate test (Fig. 1D) of UCP2 overexpressing animals

On day 1, there was no statistical difference with regard to the pre- and post-ethanol reactions between the two groups. However, there was a tendency for transgenic mice to show shorter hot-plate latency after the ethanol treatment, whereas this latency was longer than the pre-treatment result in the wild-type controls. On day 5, pre- and post-ethanol latencies were significantly shorter in the transgenic mice ($P < 0.01$ and 0.05, respectively), but not in the controls compared to the first pre-ethanol reaction. In the test before the last ethanol treatment, the transgenic animals showed significantly shorter reaction times than the control mice ($P < 0.05$). There was no significant difference between the knockout and control groups on either day 1 or day 5. However, the post-ethanol latencies were significantly longer in both groups ($P < 0.01$; $P < 0.05$).

Blood alcohol levels did not differ between transgenic and wild-type animals 1 hr after injection of the dose (3.5 mg/kg) used for the hypnotic tests: *UCP2* overexpressing mice: 317 ± 27 mg/10 mL, wild-type littermates: 305 ± 19 mg/10 mL; *UCP2* knockout mice: 345 ± 33 mg/10 mL, wild-type littermates: 326 ± 23 mg/10 mL.

4. Discussion

We observed that increased levels of *UCP2* expression corresponded with decreased ethanol sensitivity and increased ethanol tolerance. The employed paradigm of ethanol treatment is a model of binge drinking and acute intoxication rather than of level drinking. A rapid decrease in tolerance in the case of ethanol infusion or dosage on a daily basis for more than 3 days has been reported [11]. The re-sensitization that we saw on days 4 and 5 most likely is associated with neurotransmitter receptor up-regulation as described in the case of the repeated administration of high

ethanol doses for GABA receptors [12,13]. Increased sensitivity in *UCP2* overexpressing animals and its absence in *UCP2* knockout mice suggest that adaptation of receptor expression may be altered in response to ethanol administration due to UCP2 expression levels. Another possible explanation for altered sensitivity is that UCP2 enhances the metabolism and/or elimination of alcohol. While there was a trend for faster clearance of ethanol in *UCP2* overexpressing animals, it was not found to be significantly different from that of wild-type animals. Nevertheless, the tolerance and re-sensitization patterns in the different transgenic and control mice may be the consequence of mechanisms relating to ethanol metabolism, as well. Clinical and experimental data have shown that the initial sensitivity to alcohol and the development of alcohol tolerance are negatively correlated with levels of subsequent ethanol intake [14–17]. Thus, it is not unlikely that UCP2 expression levels will have a significant impact on ethanol preference.

UCP2 overexpressing mice had a shorter latency on the hot plate after ethanol treatment than their wild-type littermates. Differences in the opposite direction were observed between the *UCP2* knockout mice and their wild-type controls; however, this did not reach the level of statistical significance. Because there were no significant differences in the hot-plate tests of transgenic and control animals before ethanol treatment, it is tempting to suggest that it may be the induction of UCP2 activity by ethanol that affects pain sensation. It should be noted that a major limitation of these experiments is the fact that the *UCP2* gene-trap animals are from a different genetic background than those expressing human *UCP2*. Thus, direct comparisons between overexpressors and knockout animals have not been possible. In fact, it is known that differences in ethanol preference, pain threshold vary between mouse strains [18,19].

Ethanol tolerance and sensitivity as well as pain sensation are mechanisms involving both peripheral and central tissues. UCP2 expression in subcortical areas [5,6] and its expression in primary sensory afferents [6] suggest that UCP2 can regulate the signaling flow of pathways associated with ethanol tolerance and pain sensation at multiple brain sites. In addition, however, it cannot be excluded that UCP2 and UCP3 expression in peripheral tissues, such as muscle, could have contributed to the observed phenotypes in transgenic and gene-trap knockout animals. However, muscle impairment of either *UCP2* or *UCP3* knockout or overexpressing animals has not been reported [20–24]. In fact, in the present study a negative correlation was observed between UCP2 expression and time lapse on the hot-plate test of ethanol-exposed animals, suggesting that muscle impairment does not play a role in these events.

Functioning UCP2 may have multiple effects on cellular activity, including control of ATP and calcium homeostasis, pH levels, and free-radical production [5,20,21]. Our data suggest that UCP2 enhances protective peripheral and central neural mechanisms in acute ethanol intake revealing UCP2 as a novel target for drug development for ethanol intoxication.

Acknowledgments

This work was supported by NIH Grants MH-59847 and NS-41725, the Wyeth-Ayerst Research Institute, and a grant from the German Research Society (DFG SP 432/1-1). We are indebted to Rainer Spanagel for his helpful comments and to Marya Shanabrough and Erzsebet Borok for their excellent technical support.

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