Curcumin ameliorates ethanol-induced memory deficits and enhanced brain nitric oxide synthase activity in mice

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Abstract

Ethanol consumption has well-known deleterious effects on memory. However, the mechanism by which ethanol exerts its effects on memory has received little attention, which has retarded the identification and development of effective therapeutic strategies against ethanol toxicity. The aim of this study was to explore the neuronal mechanisms underlying the protective action of curcumin, a natural polyphenolic compound of Curcuma longa, against ethanol-induced memory deficits. Adult mice were pretreated with curcumin (40 mg/kg, i.p.) before administration of ethanol (1 g/kg, i.p.) for the memory acquisition measurement, or were sacrificed 30 min later for evaluation of regional brain differences in the nitric oxide synthase (NOS) activity and nitric oxide (NO) concentration. The results showed that pretreatment with curcumin significantly ameliorated the memory deficits resulting from acute ethanol administration to mice in the novel object recognition and inhibitory avoidance tasks. Furthermore, acute ethanol treatment increased the NOS activity and NO production in brain regions associated with memory including prefrontal cortex (PFC), amygdala and hippocampus, while this enhancement was suppressed by pretreatment with curcumin. Taken together, these results suggest that the protective effects of curcumin on acute ethanol-induced memory deficits are mediated, at least in part, by suppressing NOS activity in the brain of mice. Thus, manipulation of the NOS/NO signaling pathway might be beneficial for the prevention of ethanol toxicity.

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1. Introduction

It is well known that ethanol consumption can induce cellular damage in cerebral cells and has adverse effects on memory. Although many studies have examined the effects of chronic or acute alcohol consumption on memory, the neuronal mechanism by which ethanol exerts its toxic effects on memory impairments remains elusive. There is evidence showing that chronic or excessive exposure to ethanol affects the generation of nitric oxide (NO) (Matsuo et al., 2001; Syapin, 1998). Such an alteration in NO production in the brain as induced by ethanol may play an important role related to these neuronal dysfunctions as indicated by various behavioral modifications (Deng and Deitrich, 2007; Liu et al., 2005).

In recent years, polyphenol, obtained from certain medicinal plants and possessing high safety margin has been identified as a potential pharmacotherapy in the treatment of neuronal dysfunction (Guo et al., 2007; Schulze et al., 2005). Notably, curcumin, as the biologically active polyphenolic component of Curcuma longa (a plant in Asia), exhibits a variety of pharmacological activities including anti-inflammatory, antioxidant, immunomodulatory and antcinergic activities (Aggarwal and Harikumar, 2009; Maheshwari et al., 2006). Curcumin has been reported to be a multi-target natural compound which may modulate numerous signaling pathways, intracellular molecules and key enzymes (Zhou et al., 2011). Our previous data demonstrated that pretreatment with NMDA receptor antagonists can inhibit the antidepressant-like effects of curcumin in the forced swim test, which suggest the involvement of glutamic system by curcumin (Zhang et al., 2013). We also found that chronic treatment with curcumin could activate MAPK/ERK-dependent brain-derived neurotrophic factor expression in the amygdala of mice (Zhang et al., 2012). In recent years, curcumin has been implicated to be neuroprotective in a variety of neurodegenerative disorders such as Alzheimer’s disease (AD), Parkinson’s disease (PD) and cerebral ischemia (Pan et al., 2012; Wang et al., 2005; Yang et al., 2005). For example, curcumin has been demonstrated to penetrate into the brain and protect the substantia nigra cell death from 6-OHDA neurotoxicity (Zbarsky et al., 2005). However, the potential effects and underlying molecular mechanisms of curcumin on the
improvement of acute ethanol-induced neuronal toxicity and memory impairments in mice are not yet completely defined.

Nitric oxide (NO) has been proposed to act as a neuronal messenger molecule in the brain (Dawson and Snyder, 1994). NO synthase (NOS) is classified into three major isoforms, neuronal (nNOS), endothelial (eNOS), and inducible (iNOS). nNOS is the predominant enzyme synthesizing NO in the brain (Zhou and Zhu, 2009). It has been reported that NO affects many physiological functions including neurogenesis, learning and memory (Boehning and Snyder, 2003). Administration of l-arginine, a substrate to NO in the brain, raised the concentration of NO and promoted the memory formation in rats (Vanaja and Ekambaram, 2004). In contrast, NO has also been demonstrated to play a major role in ischemic damage and neurotoxicity (Dawson et al., 1996; Huang et al., 1994). Some studies have demonstrated that ethanol selectively affects NO production in different brain cells, and modulation of NOS is suggested to be involved in alcohol-induced behavioral modifications (Deng and Deitrich, 2007; Shih et al., 2001). As it has been reported that ethanol could exert its toxicity by alternating NOS activity and NO production in the brain (Davis and Syapin, 2005; Syapin, 1998). We speculate that the neuroprotective effects of curcumin may be related to manipulation of the NOS/NO pathway, a crucial mediator that is thought to be potentiated in the neuronal damage.

Accordingly, the present study was designed to explore the effects of curcumin on acute ethanol-induced memory deficits and the possible involvement of NOS activity in mediating the neuroprotective effects of curcumin. To accomplish this goal, the effects of treatment of acute ethanol and curcumin were evaluated with the use of a novel object recognition task and an inhibitory avoidance test, a predictive refutation model widely used for assessing memory acquisition. In addition, differences in activation of NOS/NO pathways within specific brain regions following acute ethanol administration in mice were examined.

2. Materials and methods

2.1. Animals

Ninety-two male Kun-Ming mice weighing 22–25 g were obtained from the Shandong University Animal Centre. All procedures were approved by the Shandong University Animal Care and Use Committee and were performed in accordance with the National Institutes of Health guide to care and use of laboratory animals. Mice were housed in groups of four per cage in standard Plexiglas cages and maintained on a 12 h light/dark cycle at room temperature (22–26 °C). Standard laboratory food and water were available ad libitum except during experimental procedures. Animals were allowed to acclimatize to the laboratory conditions for 7–8 days prior to the behavioral procedures which were carried out in the light phase of the photoperiod. All efforts were made to minimize the pain and numbers of the animals used in these experiments.

2.2. Drugs and treatment

Curcumin (Sigma, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA) at a concentration of 10 mg/ml and the concentration of DMSO did not exceed 0.1% of the total volume. Ethanol (95%, v/v) was diluted in physiological saline (NaCl 0.9%) to a concentration of 20% (v/v). In all experiments, ethanol or saline was administered intraperitoneally (i.p.) in a constant volume at 30 min prior to behavioral testing.

Animals were randomly allocated to one of the following four groups with N = 10–12/group: (a) vehicle (corresponding vehicles: physiological saline or 0.1% DMSO, 10 ml/kg), (b) ethanol (1 g/kg), (c) curcumin (40 mg/kg) followed by ethanol, (d) DMSO (10 ml/kg) followed by ethanol. Dose and route administration schedules of curcumin used in the present experiment were chosen as based on previous results (Bhutani et al., 2009). In the case of any significant alterations in brain nNOS activity following treatment with ethanol, curcumin was injected at 24, 5, 1 and 0.5 h prior to the injection of ethanol. To habituate mice to the intraperitoneal injection, all mice were administered saline (10 ml/kg) daily for three days prior to the experiment.

2.3. Novel object recognition task

Animals were tested in the novel object recognition (NOR) task as described previously with minor modifications (Ennaceur and Delacour, 1988; Levallet et al., 2009). Briefly, the chamber used consisted of a Plexiglas cage (60 × 40 × 40 cm) with an exchangeable floor. The objects to be discriminated were water-filled plastic bottles, 8 cm high × 5 cm in diameter, covered with white masking tape. The animals were placed individually in the empty chambers for a 1 h habituation period at 24 h prior to cognitive testing. On the test day, each mouse was placed in the same chamber for a 10 min re-habituation period. Mice were then briefly returned to their home cage to permit the introduction of the objects. The test session consisted of two consecutive phases (each 3 min in duration) separated by 1 h intervals. Ethanol or saline was injected (i.p.) immediately before the first phase (sample phase) to evaluate the effects of ethanol on memory formation. During the sample phase, each mouse was placed in the chamber with two identical objects (placed in the right and left corners of the chamber), and the time spent exploring each object was recorded. After a delay of 1 h, the animals were returned to the chamber with one of the familiar objects replaced with a novel object (in the same location). During this testing phase, exploration of the two objects was recorded as previously described in the sample phase. The chamber and the objects were cleaned with 20% v/v ethanol prior to each phase to remove any olfactory cues. For each mouse the objects and the side of novel object presentation were counterbalanced and randomly permuted.

Exploratory behavior was defined as sniffing, touching and moving vibrissae while directing their nose towards the object within the distance of 1 cm. The basic behavioral measurement was the mean time(s) spent exploring each object. Additionally, a discrimination ratio which represents the ability to discriminate the novel from familiar object: (novel/(novel + familiar time)) was calculated.

2.4. Inhibitory avoidance test

The effect of ethanol on non-spatial long-term memory was investigated using a step-down inhibitory avoidance test based on previously described procedures with some modifications (Sakaguchi et al., 2006). The apparatus consisted of a cage (23 × 21 × 22 cm) with a grid floor linked to shocker units. A wooden platform (4 × 4 × 4 cm) was fixed at the center of the grid floor. During the training session, 30 min after drug treatment, each mouse was placed on the platform and the step-down latency (SDL) was automatically recorded. When the mouse stepped down (four paws on the grid), they received a 0.3-mA scrambled foot shock for 3 s. The retention session was performed 24 h later, with a similar procedure except that no electric shock was applied after stepping down. Each mouse was placed on the platform, and the SDL was recorded. The upper cut-off time was set at 300 s.

2.5. Open field

Spontaneous locomotor activity was assessed in the open field test described previously (Walsh and Cummins, 1976). The mice were individually placed in a plywood apparatus (45 cm in diameter × 30 cm high) with a floor divided by masking tape markers into 19 equal squares. Horizontal locomotor activities (number of segments crossed
with the four paws) and vertical exploratory activities (number of time rearing on the hind limbs) were calculated during a period of 5 min.

2.6. Determination of NOS activity and NO concentration

Animals were decapitated 30 min after the last injection of the drugs or their vehicles. The whole brain was removed quickly, and the PFC, amygdala and hippocampus were carefully dissected and homogenized in ice-cold buffer solution. The homogenates were centrifuged at 15,000 g for 30 min (at 4 °C) and supernatants were assayed for NOS activity according to the protocol of the NOS assay kit (Yin et al., 2007). Briefly, enzymatic reactions were conducted with the supernatant, substrate L-arginine and cofactors (reaction reagents in the kit) at 37 °C for 15 min and then terminated by a stop buffer from the kit. The NO metabolite nitrite/nitrate were assayed with the Griess reagent from the kits as determined at 530 nm with a spectrophotometer to reflect the activity of NOS. One unit of the enzyme activity was defined as the amount that catalyzed the formation of 1 nM NO from L-arginine per minute per milligram of protein at 37 °C. The protein content of the supernatant was measured by Coomassie brilliant blue methods.

Levels of nitrite/nitrate, the stable end products of NO, were measured using a NO kit with a modification of a previously described assay (Xiong et al., 2001). Briefly, nitrate was converted to nitrite with aspergillus nitrite reductase, and the total nitrite was measured with the Griess reagent. After a 10 min incubation period at room temperature, the absorbance was determined at 543 nm using a spectrophotometer. All samples were done in duplicate. Commercial assay kids of NOS and nitrite/nitrate were obtained from Nanjing Jiancheng Biological Medical Engineering institute (Nanjing, P. R. China). In this study, PFC, amygdala and hippocampus of each mouse were separately analyzed.

2.7. Data analysis

The step-down latency results in the inhibitory avoidance test were expressed as median ± quartiles, and they were analyzed by Kruskal–Wallis non-parametric one-way analysis of variance (ANOVA) followed by a two-tailed Mann–Whitney U-test. The rest of the values were expressed as the means ± S.E.M. One-way ANOVA was used to establish the differences among groups of animals treated with vehicle or drugs alone and was followed by the Newman–Keuls test for post-hoc comparisons. Other data were analyzed statistically by two-way ANOVA for multiple comparisons with pretreatment or co-administration, and post-hoc Bonferroni test was conducted to determine differences between specific groups. p values less than 0.05 (p < 0.05) were considered as indicative of significance.

3. Results

3.1. The effects of acute ethanol and curcumin treatment on novel object recognition

Results of memory measurement were analyzed by two-way ANOVA (ethanol × curcumin). During the sample phase of the object recognition task, mice of all groups showed similar exploration times with the identical sample objects (F3, 44 = 1.03, NS). In contrast, ANOVA showed a significant effect in the factors of pre-treatment [curcumin or DMSO; F(1, 22) = 12.56, p < 0.05], treatment [ethanol or saline; F(1, 22) = 11.27, p < 0.05] and the pre-treatment × treatment interaction [F(1, 23) = 10.76, p < 0.05] on exploration time in the novel object recognition test. Post-hoc analyses indicated that pre-treatment with curcumin significantly increased the amount of time directed to novel object exploration during the testing phase in ethanol-treated mice, which suggest that curcumin but not of DMSO reversed the ethanol-educed memory deficits (p < 0.05) (Fig. 1). In addition, ethanol and curcumin had no effect on the total object exploration time during the novel object tasting phase.

3.2. The effects of acute ethanol and curcumin treatment on inhibitory avoidance test

During the training session in the step-down inhibitory avoidance test, there was no difference in the step-down latency among groups [Kruskal–Wallis non-parametric ANOVA, H(3) = 2.9, p > 0.05, n = 12] (Fig. 2A). However, in the retention session (performed 24 h later), the step-down latency was significantly decreased in ethanol-treated group compared to the vehicle treated group [Kruskal–Wallis non-parametric ANOVA, H(3) = 15.3, p < 0.05, n = 12]. Moreover, while pre-treatment with curcumin, at a dose of 40 mg/kg, significantly prevented the decreased latency in mice treated with ethanol [Kruskal–Wallis non-parametric ANOVA, H(3) = 11.7, p < 0.05, n = 12] (Fig. 2B).

3.3. The effects of acute ethanol and curcumin treatment on open field test

Horizontal and vertical locomotor activities were measured in the open-field test. There were no overall significant differences for the number of crossings (F3, 43 = 0.62, NS) and rearings (F3, 43 = 0.58, NS) among groups (Fig. 3).
The concentration of nitrite in PFC, amygdala and hippocampus was measured by converting nitrate into nitrite to reflect the production of NO. In parallel with the changes in NOS activity, ANOVA revealed the effects of pre-treatment [curcumin or DMSO; PFC: F1, 22 = 10.31, p < 0.05; Amygdala: F1, 22 = 9.73, p < 0.05; hippocampus: F1, 22 = 12.19, p < 0.05], treatment [ethanol or saline; PFC: F1, 22 = 9.72, p < 0.05; Amygdala: F1, 22 = 10.36, p < 0.05; hippocampus: F1, 22 = 13.01, p < 0.05] and the pre-treatment × treatment interaction [PFC: F1, 22 = 10.48, p < 0.05; Amygdala: F1, 22 = 9.34, p < 0.05; hippocampus: F1, 22 = 11.93, p < 0.05] on levels of NOS activity in different brain regions. Post-hoc analyses indicated that the nitrite/nitrate concentration was significantly increased by ethanol treatment in all brain areas sampled compared with that of vehicle-treated mice, while pre-treatment of curcumin significantly prevented this enhancement induced by ethanol (p < 0.05). In contrast, DMSO did not affect the enhancement of nitrite/nitrate concentrations of ethanol-treated groups (p > 0.05) (Fig. 5). These results indicate that curcumin could prevent the increase in NO production induced by acute ethanol treatment.

4. Discussion

The present study provides behavioral and neurochemical evidence demonstrating that curcumin, a natural polyphenolic compound present in C. longa, is a potent protector against acute ethanol-induced memory deficits via suppressing the NOS/NO signaling pathway. We demonstrated first, that acute ethanol treatment increased NOS activity and NO synthesis in the PFC, amygdala and hippocampus of mice. More importantly, pre-administration of curcumin significantly suppressed activity of the NOS/NO pathway, and thus improved memory performance in ethanol-treated mice as evaluated in object recognition and inhibitory avoidance tasks. These results indicate that NOS/NO signaling pathway is involved in the protective effects of curcumin upon acute ethanol-induced memory deficits in mice.

4.1. Novel object recognition task

Effects of chronic and acute ethanol consumption on memory have been assessed in many animal models in recent years (Heffernan, 2008; White, 2003), although very few studies have examined the neural correlates of ethanol-induced memory impairments. The novel object recognition task we used here is a two-trial test of non-spatial short-term memory based on the spontaneous preference of rodents for novel objects, which has previously been...
Fig. 4. Effects of curcumin (40 mg/kg, i.p.) on NOS activity in PFC (A), amygdala (B) and hippocampus (C) of ethanol-treated (1 g/kg, i.p.) mice. All values are presented as means ± SEM from ten to twelve animals per group. *p < 0.05 when compared to the vehicle group and #p < 0.05 when compared to the ethanol group.

Fig. 5. Effects of curcumin (40 mg/kg, i.p.) on the nitrite/nitrate concentration in PFC (A), amygdala (B) and hippocampus (C) in ethanol-treated (1 g/kg, i.p.) mice. All values are presented as means ± SEM from ten to twelve animals per group. *p < 0.05 when compared to the vehicle group and #p < 0.05 when compared to the ethanol group.
shown to be sensitive to drugs that produce impairment or enhancement of memory (Puma et al., 1998; Woolley et al., 2003). Consistent with previous studies, the present data showed that all groups explored the two identical objects to a similar extent, which indicate that rats had no spatial preference for either of the two object positions. In contrast, following a 1 h interval, ethanol treated mice tend to explore the novel objects to a lower extent than the familiar objects as compared with vehicle-treated control groups, suggesting that acute ethanol produced a marked long-term deficit in recognition memory. Interestingly, pre-treatment with curcumin resulted in a significantly greater novel object preference than ethanol-treated mice, which indicate that curcumin prevented deficits in discriminative memory produced by ethanol. Further, ethanol and curcumin treatment did not interfere with the total object exploration time during the novel object tasting phase, which confirms a shift in relative time directed to the novel object.

4.2. Inhibitory avoidance test

Another test employed in this study for assessing non-spatial long-term memory assessment was the inhibitory avoidance task which reflects the motivation for inhibitory avoidance based on the fear of foot shock (Kameyama et al., 1986). The present results showed that the step-down latency was decreased in ethanol-treated mice as compared to control animals, which indicated that ethanol caused impairments on memory retention. In this task, of greater significance was the finding that pre-administration of curcumin was effective in preventing this memory deficit. Based on the findings from these two tasks, it could be concluded that treatment with curcumin ameliorates memory impairments in this ethanol-treatment model. In addition, it should be noted that in the open field test, mice treated with curcumin and/or ethanol demonstrated a similar number of crossings (locomotor activities) and rearing (exploratory activities) as that of control animals, which indicated that the effects of both ethanol and curcumin on memory in this study are dissociated from any alteration of ambulation activity.

4.3. NOS/NO pathway in the amelioration effects of curcumin in memory deficits

Science ethanol pretreatment produced disruption of short-term and long-term memory performance on these two memory tests, we sought to determine if this behavioral effect was associated with some specific neural response in the brain. It is generally considered that NO is an important molecule associated with both physiological and pathological brain events (Davis and Sypain, 2005). Recent studies also reported that the NOS/NO pathway played an important role in ethanol modification of brain structure and function (Kanbak et al., 2013; Kashem et al., 2012). Therefore, effects of ethanol on NO production may likely be involved with ethanol-induced memory impairments. It is well known that prefrontal cortex (PFC), hippocampus and amygdala are the key brain regions involved in memory processes. Previous studies have reported that these three brain regions were involved in modulating consolidation of object recognition and inhibitory avoidance memory (Benetti and Izquierdo, 2013; Ferry and McGough, 2008; Mello-Carpes and Izquierdo, 2013; Rozendaal et al., 2008). In the present study, the activity of NOS and the concentration of NO were enhanced within these three brain regions of ethanol treated mice, suggesting a neurobiological mechanism associated with ethanol modification of memory. The extent and direction of effects of NO depend on the dose of exposure and cell type. For example, low physiological concentrations of NO can inhibit apoptosis, but higher concentrations of NO may be toxic (Kim et al., 2001). Therefore, ethanol-induced impairments of memory might be mediated by enhanced overproduction of NO in these brain regions. Interestingly, curcumin has been recently shown to exert a protective effect upon brain edema after hypoxic–ischemic brain damage by down-regulating NOS activity in rat (Yu et al., 2012). In the present experiment, we focused our interest on the memory process after ethanol consumption, and found that pre-administration of curcumin decreased NOS activity and NO production levels in the PFC, hippocampus and amygdala in ethanol-treated mice, suggesting that the NOS/NO signaling pathway may represent an important molecular sequence of events exerted by curcumin to prevent these memory deficits. Accordingly, our results reveal a strong link between memory deficits and NO production and neuroprotection of these processes as mediated by curcumin.

5. Conclusion

In summary, the results of the present study provide strong evidence that acute ethanol enhances NOS activity and NO concentration in specific mammalian brain regions associated with learning and memory formation. The changes of NOS activity and NO concentration observed in ethanol-treated mice may have behavioral implications for learning and memory deficits. More importantly, the amelioration in memory deficits by curcumin in ethanol-treated mice was accompanied with a suppressed activity of nNOS/NO signaling pathway in these brain regions. Taken together, these findings revealed that curcumin may function as a potent protector against acute ethanol-induced memory deficits.

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