Ethanol Upregulates iNOS Expression in Colon Through Activation of Nuclear Factor-kappa B in Rats

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Background: Alcohol inhibits colonic motility but the mechanism is unknown. The goal of this study was to test the possibility that nuclear factor-kappa B (NF-κB) is involved in the upregulation of inducible nitric oxide synthase (iNOS) expression induced by ethanol in colon.

Methods: The isometric contraction of longitudinal muscle strips of proximal colon (LP) was monitored by polygraph. Western blot analysis was used to measure the amount of iNOS and IκB in the cytoplasm and P65 in the nucleus. Immunohistochemistry was applied to locate iNOS in colon.

Results: Ethanol (87 mM) inhibited the contraction of LP. Pretreatment of S-methylisothiourea (SMT) (1 mM), a specific iNOS inhibitor, Pyrrolidine dithiocarbamate (PDTC) (10 mM) and BAY11-7082 (10 mM), specific inhibitors of NF-κB significantly reversed the inhibitory effect of ethanol on LP contraction. Ethanol increased the amount of iNOS and content of NO in colon, and these effects were attenuated by pretreatment of PDTC. Following ethanol administration, the amount of IκB in the cytoplasm decreased, but that of P65, the subunit of NF-κB in the nucleus, increased. The iNOS was located in the cell body of myenteric plexus in colon.

Conclusion: Ethanol inhibited the contraction of LP in colon mainly through activation of NF-κB, the subsequent upregulation of iNOS expression and increase of NO release in myenteric plexus.

Key Words: Colon Motility, iNOS, NF-κB, Ethanol.
with free access to water before experiments. All experimental procedures were approved by the Ethics Committee for Research on Animals, Shandong University School of Medicine.

**Muscle Strips Preparation**

The method has been described elsewhere with some modification (Wang et al., 2007). Briefly after an animal was killed, a segment of proximal colon (1 cm from caecum) was removed. The segment was opened along the mesenteric border and the resulting rectangular sheet was pinned flat (mucosa up) in a silgard lined Petri dish filled with oxygenated Krebs solution. Muscle strips (8 × 3 mm) were then cut parallel to the longitudinal muscle fibers, these were designated LP. Finally, the mucosa layer on each strip was carefully scraped away.

**Recording of the Contraction of Muscle Strips**

The muscle strip was suspended in tissue chamber containing 5 ml Krebs solution (37°C) and bubbled continuously with 95% O₂ and 5% CO₂. One of the narrow ends of the strip was tied to a hook at the bottom of the chamber. The opposing end was connected to an external isometric force transducer (JH-2B, Instrument Company of Chengdu, Chengdu, China). The temperature of the chamber was kept at 37 ± 0.5°C. Contraction of colonic strips (under an initial tension of 1 g) in the tissue chambers was recorded using a polygraph system (SMUP-PC, Fudan University, Shanghai, China). To stabilize background contractions after excision, the muscle strips were initially equilibrated in the tissue chamber for 60 minutes, during this period the strips were washed by the fresh Krebs solution (37°C) every 10 minutes. Ethanol was administered after the spontaneous motility of colonic strips had become stable. The average muscle tension over 10-second periods was calculated using MF Lab software (Fudan University, Shanghai, China) which integrating the tension—time trace over each recording period. Each muscle strip was exposed to ethanol only once and tension was recorded continuously for 20 minutes after ethanol administration. In experiments that investigated the mechanism underlying the effects of ethanol, some specific inhibitors were pretreated for 30 minutes before administration of ethanol.

**Chemicals and Solutions**

Pyrrolidine dithiocarbamate (PDTC, a NF-κB inhibitor), S-methylisothioura (SMT, a specific inhibitor of iNOS) were purchased from Sigma Company (St. Louis, MO), BAY11-7082 (an NF-κB inhibitor) was purchased from Beyotime Institute Biotechnology of (Shanghai, China).

The Krebs solution was composed of the following reagents (mM): NaCl 120.6, KCl 5.9, CaCl₂ 2.5, KH₂PO₄ 1.2, MgCl₂ 1.2, NaHCO₃ 15.4, and glucose 11.5.

Ethanol, PDTC, and SMT were diluted into Krebs solution, BAY was diluted into dimethyl sulfoxide (DMSO).

**Western Blot Procedures**

The segments of proximal colon were removed and the mucosa layer on each strip was carefully scraped away. The tissue was treated with ethanol for 2 minutes before it was prepared for Western blot. The body homogenates were electrophoresed and transferred to nitrocellulose membranes. Membranes were blocked for 1 hour at room temperature in blocking buffer (5% nonfat dry milk, TTBS), washed in TTBS (0.1% Tween 20, 50 mM Tris, and 150 mM NaCl), and incubated overnight with rabbit anti-iNOS antibody (1:500, sc 651; Santa Cruz, CA) or rabbit anti-NF-κB antibody (1:500, sc 8008; Santa Cruz) or rabbit anti-IκB antibody (1:800, sc 371; Santa Cruz). After washing 3 times, membranes were incubated for 1 hour at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:20,000). Finally, immunoreactive proteins were detected by ECL plus (Millipore, Bedford, MA).

**Nuclear and Cytoplasmic Protein Extraction**

The nuclear and cytoplasmic protein extraction kit was bought from BioTeke Corporation (Beijing, China), which included cytoplasmic extraction reagent A (CER A) and nuclear extraction reagent B (NER B). The tissue was treated with ethanol for 20 minutes before it was prepared for Western blot. Sample of proximal colon (0.1 g) was homogenized in 0.5 ml of CER A. After vortexing for 10 seconds, the homogenates were centrifuged at 15,000 rpm for 10 minutes at 4°C, the supernatants containing cytoplasmic proteins were collected and stored at −80°C. The sediments was dissolved by rotating for 60 minutes at 4°C in 0.5 ml of a PMSF buffer added NER B. After vortex and centrifugation at 15,000 rpm for 10 minutes at 4°C, the supernatants were collected as nuclear extracts and stored at −80°C.

**Measurement of NO Levels in Colon**

The segments of proximal colon were removed and the mucosa layer was carefully scraped away. The tissue was treated with ethanol for 20 minutes before it was prepared for the measurement of NO content. The samples were thawed, weighed and homogenized in 1:9 w:v in 0.9% saline. The homogenates were then centrifuged at 1,000 r/min for 5 minutes at 4°C, the supernatant was taken for NO assay and total protein determination. NO was assayed spectrophotometrically by measuring total nitrate plus nitrite (NO₃⁻ plus NO₂⁻) and the stable end products of NO metabolism. The procedure that nitrate was enzymatically converted into nitrite by the enzyme nitrate reductase was followed by quantization of nitrite using Griess reagent at the absorbance of 550 nm (Tarpey et al., 2004). The level of NO was expressed as μmol/g protein.

**Immunohistochemistry**

The segments of proximal colon were removed and treated with ethanol for 20 minutes. Immunohistochemistry for nitric oxide synthase (iNOS) was performed on 4-μm-thick paraffin-embedded sections from thickness of colon in rats, utilizing the labeled streptavidin-biotin method using a Two-Step IHC Detection Reagent (ZSGB-BIO, Beijing, China). The paraffin-embedded sections were heated for 2 hours at 120°C, deparaffinized in xylene, and rehydrated through graded ethanol at room temperature. After 3 rinses in PBS, microwave accentuation was used for 10 minutes. Then after 2 rinses in PBS, sections were put in a humid chamber for 15 minutes. Next, sections were treated for 40 minutes at room temperature with 2% bovine serum albumin, and incubated overnight at 4°C with primary antibodies, rabbit anti-iNOS antibody (diluted 1:200 in PBS, sc 651; Santa Cruz). After the sections were washed, they were incubated with biotinylated goat anti-rabbit serum (ZSGB-BIO, Beijing, China) for 30 minutes at room temperature. The sections were then washed and then treated with HRP-labeled streptavidin-complex (ZSGB-BIO) for 30 minutes at room temperature. After several rinses, peroxidase was revealed by a 3,3-diaminobenzidine tetrahydrochloride substrate kit (ZSGB-BIO). Finally, the sections were weakly counterstained with hematoxylin.

In negative controls, the sections were incubated with PBS instead of primary antibody.

**Data Analysis**

**Muscle Strip Contraction**

The mean value of the integration in 3 minutes before the treatment of ethanol was considered as...
the baseline. The mean integration at different time point following ethanol treatment was divided by that of baseline, and the resultant value, $R$, was regarded as the change of contraction of the muscle strips induced by ethanol with and without pretreatment of inhibitors. The $R$-value of baseline is 1. The value of $R$ was presented as mean ± SEM, with $n$ indicating the number of rats. Student’s $t$-test was used to analyze the effect of antagonist. One way analysis of variance (ANOVA) followed by Dunnett’s test was used to analyze the effect of ethanol. $p < 0.05$ was considered to be significantly different.

**Western Blot.** The Western blot results were quantified using Scion Image software (Scion Corp., Frederick, MD), background band was subtracted and the band were expressed as relative protein amounts compared to β-actin (cytoplasmic protein) or c-jun (nuclear protein). Statistical comparisons were made using ANOVA, $p < 0.05$ was considered to be significant.

**NO Content.** Results were expressed as mean ± SEM. Statistical comparisons were made using ANOVA, $p < 0.05$ was considered to be significant.

**RESULTS**

**Effect of Ethanol on LP Contraction**

Ethanol (87 mM) inhibited the spontaneous contraction of LP from rats (Fig. 1A). The contraction of LP decreased immediately after the ethanol administration (Fig. 1A). At 5, 10, 15, and 20 minutes after ethanol administration, R of LP decreased from 1 (baseline) to 0.80 ± 0.02, 0.81 ± 0.03, 0.78 ± 0.03 and 0.79 ± 0.01 respectively ($n = 8$, $p < 0.05$, Fig. 1B). Ethanol (17, 35, 57, 87, 130 mM) dose-dependently inhibited contraction of LP from rats (Fig. 1C). After the treatment with the 5 doses, R of LP decreased from 1 (baseline) to 0.97 ± 0.02, 0.86 ± 0.03, 0.80 ± 0.03, 0.77 ± 0.03, and 0.79 ± 0.01 respectively ($n = 8$, $p < 0.05$, Fig. 1C).

**Effect of PDTC, BAY, and SMT on Ethanol-Induced Inhibition on LP Contraction**

Ethanol (87 mM) inhibited the spontaneous contraction of LP from rats. PDTC (10 mM) and BAY (10 mM), 2 specific inhibitors of NF-κB (Mendez-Samperio et al., 2009), SMT (10 mM), a specific inhibitor of iNOS (Su et al., 2007), partially reversed the inhibitory effect of ethanol on the contraction of LP ($n = 8$, $p < 0.05$, Fig. 2A–C).

**Expression of iNOS, I-κB, and NF-κB Following Ethanol Treatment With and Without the Pretreatment of PDTC**

Ethanol (35, 57, 87, 130 mM) increased the expression of iNOS in colon (Fig. 3A). Pretreatment of PDTC (10 mM) significantly attenuated the increase of iNOS expression following ethanol (Fig. 3B).

Ethanol (87 mM) significantly decreased the amount of cytoplasmic I-κB and increased nuclear P65 in colon (Fig. 4A,B). Pretreatment of PDTC (10 mM) partly reversed these change (Fig. 4A,B).

**Immunohistochemistry**

Inducible NOS immunoreactivity was expressed in myenteric plexus (Fig. 5B,D). No labeling cell was detected in sections that were treated with PBS instead of primary antibody (Fig. 5A,C).

**The NO Content in Colon**

The NO content in colon following ethanol administration increased from 1.112 ± 0.128 μmol/g (control) to 7.194 ±
0.497 μmol/g \((n = 6, p < 0.05, \text{Fig. 6})\). With the pretreatment of PDTC, the increase of NO content following ethanol administration was significantly attenuated \((n = 6, p < 0.05, \text{Fig. 6})\).

**DISCUSSION**

Our recent study indicated that ethanol inhibited the muscle strip of LP in colon in rats and this effect might be mediated by the release of NO (Wang et al., 2007). In this project, we provide more direct evidences to support the results. Following pretreatment with ethanol, iNOS expression was upregulated and release of NO was increased in colon. SMT, a more specific inhibitor of iNOS than AG, reversed the inhibitory effect of ethanol on the contraction of LP. So it is clear that the inhibition of LP contraction following ethanol administration is mainly mediated by upregulation of iNOS. Following ethanol administration, iNOS was located at the myenteric plexus in colon. So we believe that it is the cells that in ENS that released NO following ethanol administration. This data are consistent with our recent report, which indicated that TTX, the blocker of voltage dependent sodium channels on...
nerve fiber, attenuated the inhibitory effect of ethanol on LP contraction (Wang et al., 2007).

The most important control mechanism for iNOS expression is the regulation of iNOS transcription (Kleinert et al., 2003). As a family of transcription factors NF-κB seems to be a central target for activators of iNOS expression. It mediates the upregulation of iNOS following LPS, IL-1β, TNF-α, and oxidative stress in different cell types (Kleinert et al., 2003).

There are several reports about the effect of ethanol on the NF-κB activation but the results are controversial. NF-κB was activated following the release of some cytokines, such as TNF-α (Zeldin et al., 1996). Acute alcohol consumption increased the TNF-α dependent NF-κB activation but chronic alcohol consumption decreased it in the Kuffer cell of the liver (Zeldin et al., 1996). Moderate alcohol intake inhibited the activation of NF-κB in human monocytes (Mandrekar et al., 2006; Szabo et al., 2007). In contrast, acute alcohol treatment
augmented NF-κB activation and TNF-α production and inhibited IL-10 levels in the presence of complex stimulation with combined TLR2 and TLR4 ligands. Prolonged alcohol exposure also resulted in an increase in NF-κB and TNF-α production in response to TLR4 stimulation with LPS (Szabo et al., 2007). In this study, we found that the amount of cytoplasmic IκB in colon decreased and nuclear P65 increased following ethanol administration. These results indicated that NF-κB was activated (Ma et al., 2008). PDTC and BAY, specific inhibitors of NF-κB (Ma et al., 2008), reversed the inhibitory effect of ethanol on LP contraction in colon. So it is clearly that NF-κB activated by ethanol mediated the inhibitory effect of ethanol on LP.

In physiological conditions, both nNOS and iNOS are expressed in the neurons of the myenteric plexus in mouse colon (Vannucchi et al., 2002). The present data indicate that the increased expression of iNOS following ethanol is mainly expressed in the neurons of the myenteric plexus in mouse colon (Vannucchi et al., 2002). The present data indicate that the increased expression of iNOS following ethanol is mainly expressed in the neurons of the myenteric plexus in mouse colon (Vannucchi et al., 2002).

**Fig. 6.** Comparison of the NO content in the samples of proximal colon from rat pretreatment of vehicle (Krebs solution), ethanol (87 mM), and PDTC (10 mM) + ethanol (87 mM). Ethanol significantly increased the NO content in colon, PDTC significantly reversed this change. *p < 0.05 vs. vehicle control, #p < 0.05 vs. ethanol.

In conclusion, the results of present study indicated acute exposure of ethanol inhibited the LP contraction of LP in rat colon through activation of NF-κB and the subsequent expression of iNOS and release of NO from myenteric plexus.

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