Ethanol Exposure Suppresses Survival Kinases Activation in Adult Rat Testes

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(Received 12 July 2006/Accepted 30 August 2006)

ABSTRACT. The present study was designed to evaluate whether ethanol suppresses survival-signaling pathways in rat testes. Ethanol (1.5 g/kg or 3 g/kg i.p., 15% v/v in saline) was administrated to adult male rats for 10 days. Ethanol treatment significantly increased the number of TUNEL-positive cells in rat testes. Potential activation was measured by phosphorylation of Akt and Erk1/2 using Western blot analysis. Ethanol decreased the levels of activated survival kinases, pAkt and pErk1/2. The phosphorylation of Bad at Ser112 and Ser136 was decreased in ethanol-treated animals in comparison to saline-treated animals. Moreover, the interaction of pBad with 14–3–3 was decreased by ethanol exposure. In conclusion, our findings suggest that ethanol induces apoptotic cell death by suppressing the activation of survival kinases and the phosphorylation of their downstream targets in rat testes.

KEY WORDS: ethanol, survival kinase, testes.

Ethanol is among the most widely abused drugs. It is known to cause serious reproductive disorders, including testicular atrophy, accessory reproductive organ dysfunction, and infertility [1, 5, 6]. In particular, ethanol produces an oxidative stress within the testis and enhances apoptotic cell death [11, 14, 18].

The activation of survival kinases, such as Akt and Erk1/2, is a crucial step in the suppression of apoptosis and the stimulation of cell survival in response to various stresses [4, 13]. Activated survival kinases phosphorylate serine residues of Bad to attenuate its pro-apoptotic action. The phosphorylated Bad interacts with 14–3–3, which prevents Bad from binding with Bcl-x(L) at the mitochondrial membrane [12, 16, 17]. Apoptotic stimuli induce the dephosphorylation of Bad, releasing it from 14–3–3 [16, 17]. Bad releases Bax from Bcl-x(L), and Bax promotes the release of cytochrome c and the activation of the caspase cascade. Recently, we reported that ethanol decreases cell proliferation and enhances apoptotic cell death in testicular germ cells [11]. However, little data is available on the participation of signaling pathways involving the survival kinases in ethanol-treated rat testes. Thus, the present study investigated the effect of ethanol on the activation of survival kinases, Akt and Erk1/2, and their subsequent effects on downstream targets, Bad and 14–3–3, in adult rat testes.

MATERIALS AND METHODS

Animals and tissue preparation: Adult male Sprague-Dawley rats (250–300 g, n=30) were purchased from Samtako Co (Laboratory Animal Breeding Center, Korea) and were randomly divided into three groups; saline-treated group, ethanol (1.5 g/kg)-treated group, and ethanol (3 g/kg)-treated group (n=10 per group). All animal experiments were performed in accordance with the NIH Guidelines for Use of Laboratory Animals. Animals were maintained under controlled temperature (25°C) and lighting (14:10D), and allowed free access to food and water. Ethanol (1.5 g/kg or 3 g/kg, 15% v/v in saline) was administrated daily (08:00–09:00 h) intraperitoneally for 10 days, and the same volume of saline was administrated for the controls [10, 11]. Animals were decapitated, and testes were removed. Testes were fixed with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), embedded with paraffin, and sectioned for TUNEL staining.

TUNEL histochecmistry: TUNEL histochemistry was performed using the DNA Fragmentation Detection Kit (Oncogene Research Products). Sections were incubated with proteinase K (20 µg/ml) for 5 min and then washed in PBS. Sections were then incubated with a mixture of terminal deoxynucleotidyl transferase (TdT) and digoxigenin labelled deoxy nucleotide in a humidified chamber at 37°C for 1 hr. Immunostaining was carried out according to the manufacturer’s protocol. Briefly, sections were incubated with an anti-digoxigenin antibody conjugated with horseradish peroxidase for 1 hr. After sections were washed in PBS, the color reaction was performed with DAB (Sigma, St. Louis, MO, U.S.A.) solution with 0.03% hydrogen peroxidase for 3 min. Negative controls were performed using distilled water in the place of the TdT enzyme. Sections were counterstained with hematoxylin. Slides were observed under microscope, and then photographed. Quantitative analysis of testicular apoptosis in saline- and ethanol-treated groups was estimated according to Hu et al. [9]. To quantify the incidence of apoptosis, the seminiferous tubules containing three or more apoptotic cells by TUNEL stain were calculated. The apoptosis percentage was calculated by the ratio of the positive seminiferous tubules of apoptosis to the total number of seminiferous tubules in cross sections.

Western blot analysis: Tissues samples were homoge-
nized in lysis buffer (1% Triton X-100, 1 mM EDTA in PBS). The protein concentration of each lysate was determined using the bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, U.S.A.) according to the manufacturer’s protocol. Thirty micrograms of total protein was applied to each lane on to 10% SDS-polyacrylamide gels (SDS-PAGE). After electrophoresis and immunoblotting, the poly-vinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, U.S.A.) were washed in Tris-buffered saline containing 0.1% Tween-20 (TBST) and then incubated with the following antibodies: anti-phospho-Akt, anti-phospho-Erk1/2, anti-phospho-Bad (Ser136), and anti-phospho-Bad (Ser112) (diluted 1:1000, Cell Signaling, Beverly, MA, U.S.A.) as primary antibody. And the membrane was incubated with secondary antibody (1:5000, Pierce), and the ECL Western blot analysis system (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer’s protocol was used for detection. The intensity analysis was carried out using SigmaGel 1.0 (Jandel Scientific, San Rafael, CA, U.S.A.).

**Immunoprecipitation of 14–3–3**: Total protein (200 µg) was used and was pre-cleared with Protein-A agarose (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) to remove nonspecific-binding proteins. The pre-cleared samples were incubated with anti-14–3–3/antibody (Santa Cruz Biotechnology) overnight at 4°C. And then the immune complex was then precipitated with protein A/G agarose beads for 2 hr at 4°C. The protein-bead complex was then washed and collected by centrifugation. The samples were processed as described for Western blot analysis. The results are the mean of five independent experiments.

**Data analysis**: All data are expressed as mean ± S.E.M. The results in each group were compared by one-way analysis of variance (ANOVA) followed by the post hoc Dunnet test when the F value showed significant differences at *P<0.05.

**RESULTS**

We observed apoptotic cells by the TUNEL method. Within the testes of saline-treated rats, only a few TUNEL-positive cells were observed (Fig. 1A and 1D). However, the number and signal density of positive cells significantly increased in ethanol (1.5 g/kg)-treated (Fig. 1B and 1E) and ethanol (3 g/kg)-treated animals (Fig. 1C and 1F). Positive cells were detected in spermatogonia and spermatocytes. There were no positive cells in negative control (Fig. 1G). Figure 1H shows the apoptotic index in the testes of saline- and ethanol-treated animals. Ethanol administration significantly increased the apoptotic index. The apoptotic index was 3.53 ± 0.5% in saline-treated animals. While, it was 32.5 ± 1.5% and 35.5 ± 2.0% in ethanol (1.5 g/kg)-treated and ethanol (3 g/kg)-treated animals, respectively.

Upon exposure to ethanol, the expression levels of pAkt and pErk1/2 were decreased in ethanol-treated rats, in comparison to saline-treated rats (Fig. 2). The level of pAkt was 1.32 ± 0.15 in saline-treated animals, and 1.18 ± 0.09 and 1.15 ± 0.12 in ethanol (1.5 g/kg)-treated and ethanol (3 g/kg)-treated animals, respectively (Fig. 2). The level of pErk1/2 was 0.87 ± 0.07 in saline-treated animals, and 0.79 ± 0.11 and 0.78 ± 0.07 in ethanol (1.5 g/kg)-treated and ethanol (3 g/kg)-treated animals, respectively (Fig. 2). Phosphorylation of Bad on Ser136 and Ser 112 was diminished by ethanol treatment (Fig. 3). The levels of pBad Ser136 and pBad Ser112 were 1.12 ± 0.12 and 0.85 ± 0.05 in saline-treated animals, and 1.08 ± 0.07 and 0.76 ± 0.12 in ethanol (1.5 g/kg)-treated, and 1.09 ± 0.11 and 0.75 ± 0.15 in ethanol (3 g/kg)-treated animals, respectively (Fig. 3).

![Fig. 1. Representative photomicrographs of TUNEL staining and quantitative analysis of apoptosis in the testes of saline- (A and D) and ethanol-treated groups (B and E: 1.5 g/kg; C and F: 3 g/kg). TUNEL-positive cells were markedly increased in the testicular germ cells of the ethanol-treated animals. No positive signals were detected in negative control (G). Arrows indicate positive cells of TUNEL in spermatogonia and spermatocytes. D, E, and F photos indicate the square areas of A, B, and C. Scale bar: A, B, and C, 100 µm; D, E, F and G, 50 µm. The apoptotic index was calculated as the ratio of apoptosis-positive seminiferous tubules to the total number of seminiferous tubules (H). * P<0.05 (vs. control).](image-url)
Figure 4 shows the interaction of pBad and 14–3–3 by immunoprecipitation. The interaction of pBad and 14–3–3 decreased in ethanol-treated animals. The binding levels of 14–3–3/pBad were 0.87 ± 0.06, 0.75 ± 0.12, and 0.76 ± 0.09 in saline, ethanol (1.5 g/kg)-treated, and ethanol (3 g/kg)-treated animals, respectively (Fig. 4).

DISCUSSION

It is known that ethanol induces apoptotic cell death in testicular germ cells and suppresses spermatogenesis [14]. Previous our study demonstrated that ethanol significantly increased the number of apoptotic cells in both spermatogonia and spermatocytes [11]. Also, Zhu et al. [18] demonstrate that ethanol exposure enhances apoptosis of testicular germ cells, spermatogonia and spermatocytes. Furthermore, radiation induces apoptosis of germ cells, spermatogonia and spermatocytes [15]. It is known that the efficiency of spermatogenesis depends on the proliferative activity of spermatogonia and spermatocytes, and the loss of these cells during meiosis and spermiogenesis [8]. Thus, we considered that apoptotic stimuli induce apoptosis of germ cells for the prevention of spermatogenesis and suppression of reproductive function. Our result clearly confirmed that ethanol significantly increases apoptotic cell death. Also, we previously demonstrated that ethanol exposure induced the apoptosis by increasing the activation of caspase-3 in testis [11]. To better understand the specific pathways leading to cell death, we investigated the effects of ethanol on survival kinases and their downstream targets. Activation of sur-
vival kinases is important for cell growth and suppression of apoptosis [3]. This study showed that ethanol exposure decreased the activation of survival kinases, Akt and Erk1/2. The phosphorylation of Akt and Erk1/2 indicates the activation of survival kinases. It is known that Akt directly phosphorylates Bad on residue Ser136, while Erk1/2 indirectly phosphorylates Bad on residue Ser112 [2, 4, 7]. In this work, we showed that ethanol administration significantly reduced the phosphorylation of Bad on Ser136 and Ser112, a likely downstream result of reducing the amount of survival kinases Akt and Erk1/2.

When Bad is phosphorylated by activated survival kinases, it is sequestered into the cytosol in a complex with 14–3–3 and fails to interact with Bcl-x(L) at the mitochondrial membrane [16]. However, apoptotic stimuli suppress the phosphorylation of Bad and releases Bad from 14–3–3 [16, 17]. Next, Bad dimerizes with the anti-apoptotic protein, Bcl-x(L), to release Bax from Bcl-x(L). Finally, the newly liberated Bax initiates activation of caspase cascade [16, 17]. Thus, the phosphorylation of Bad and the interaction of pBad and 14–3–3 are critical for cell survival. In this study, we showed that the interaction of pBad with 14–3–3 was decreased in the presence of ethanol. Furthermore, we previously demonstrated that ethanol induces apoptotic cell death through the activation of caspase-3 in the testis [11]. In summary, our work supports that ethanol inhibits the cell survival and induces the apoptotic cell death, by decreasing in the levels of survival kinases, pAkt and pErk1/2, which, in turn, diminishes the phosphorylation level of Bad. Following this, Bad is dissociated with 14–3–3 and binds with Bcl-x(L). Finally, the newly liberated Bax initiates activation of caspase cascade and apoptotic cell death.

In conclusion, our findings suggest that ethanol induces apoptotic cell death via the suppression of survival kinases activation and the phosphorylation of their downstream targets in rat testes.

ACKNOWLEDGMENT. This work was partly supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2005–041–E00411).

REFERENCES