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Original Article

RU486 Abrogates Ethanol-Induced Apoptosis of Splenic Natural Killer Cells in a Mouse Model of Binge Drinking

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Ethanol (EtOH) decreases the number and activity of splenic natural killer (NK) cells in a mouse model of binge drinking. The fate of the lost splenic NK cells is unknown. In the present study, mice were exposed to EtOH in a model designed to represent binge drinking. The percentages of NK cells labeled with NK1.1 antibodies were determined by flow cytometry. The results demonstrated that EtOH decreases splenic NK cell percentages. In addition, EtOH suppresses NK cell percentages in peripheral blood-, bone marrow- and peritoneal cavity-derived cells. Splenic NK cell apoptosis was evaluated on sub-G1 apoptosis assay. Flow cytometric analysis demonstrated a significant increase in the percentage of apoptotic NK cells in the spleen 3-6 hr after administration of EtOH. This increase was completely blocked by the glucocorticoid antagonist RU486. RU486 also substantially blocked the increased protein expressions of Bax, Bak, cleaved caspase-3, and cleaved caspase-9 induced by EtOH. Collectively, these results indicated that EtOH-induced glucocorticoids induce splenic NK cell apoptosis via the intrinsic pathway and this may be one mechanism by which EtOH-induced stress responses lead to immunosuppression.

Keywords: ethanol, natural killer cell, glucocorticoids, apoptosis

Introduction

Binge drinking is on the rise worldwide. A binge is defined by the National Institute on Alcohol Abuse and Alcoholism (USA) as consumption of 5 and 4 drinks by men and women, respectively, in 2 hrs to produce a blood ethanol (EtOH) level of more than 0.08% (80 mg/dl). Binge drinking is remarkably common in the US. About 43% of college students reported binge drinking at least once during the previous month [1]. About 38 million US adults binge drink according to a 2010 survey by the Centers for Disease Control. The overall prevalence of binge drinking among adults in 48 states and the District of Columbia is 17.1%, resulting in an estimated annual economic cost of 168 billion dollars [2]. The overall prevalence of binge drinking among adults in Taiwan is unknown.

Natural killer (NK) cells are cardinal innate effectors of the immune system that play an important role in the development of optimal antiviral and antitumor immunity. NK cells are important in host resistance to some types of infectious agents and tumors [3].In view of the substantial prevalence of binge drinking [4] and the importance of NK cell activation in basal [5,6] and immunotherapy-mediated [7,8] resistance to cancer or viral infection, it is worth investigating the effects of EtOH on NK

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cell function.

A binge drinking model was established by Han et al. [9] in which mice were given a single dose of a 32% EtOH solution by gavage. This mouse binge drinking model was used in our previous study to demonstrate that the decreases in NK cell number and activity by EtOH are mediated by ethanol-induced glucocorticoids [10]. The fate of the lost splenic NK cells is unknown. Glucocorticoids are known to alter leukocyte trafficking [11-13] and it is possible that NK cell numbers in other lymphoid tissues increase after EtOH administration.

It has been reported that NK cells undergo apoptosis in response to glucocorticoids [14-16]. Apoptosis is mainly regulated by two interrelated signaling pathways: the extrinsic or death-receptor pathway and the intrinsic or mitochondrial pathway [17,18]. They converge on the execution pathway, which is mediated intracellularly by a cascade of cysteine proteases, termed caspases [19,20]. Therefore, additional studies to evaluate if EtOH-induced apoptosis of splenic NK cells is mediated by glucocorticoids in this mouse binge drinking model are warranted.

Materials and Methods

Mice. Female B6C3F1 mice were obtained from the Animal Center of Chung Shan Medical University (Taichung, Taiwan). Mice were certified by the supplier to be specific pathogen free with sentinel mice housed in the same room as those used in this study. Mice were kept in individual ventilated cages (IVC) at the Animal Center of Chung Shan Medical University under a 12 hr light/dark cycle and were given food and water *ad libitum*. Mice were routinely 8-12 weeks of age at the time of our experiments. Animal care and use were in accordance with the policies of the Institutional Animal Care and Use Committee of Chung Shan Medical University.

Preparation of NK effector cells. The spleen was removed from each mouse, placed in RPMI 1640 culture medium (3 ml) and pressed between the frosted ends of sterile glass microscope slides to produce a single cell suspension. A 20 µl sample of each suspension was used to quantify nucleated cells with a Coulter counter (model Zf, Beckman Coulter, Inc. Indianapolis, IN, USA), following lysis of red blood cells using Lysing and Hemoglobin Reagent (Baxter Scientific, Markham, Ontario, Canada). The splenic cells were centrifuged (250 x g for 5 min), then resuspended at 1 x 10^7 nucleated cells/ml in complete culture medium consisting of RPMI 1640 with 10% fetal bovine serum (HyClone Labs, Logan, UT, USA) and penicillin at 0.05 U/ml plus streptomycin at 0.05 mg/ml. Resident peritoneal cells were obtained by lavage of the peritoneal cavity of each mouse with 8 ml of ice cold Dulbecco's phosphate buffer without calcium and magnesium but with 10% fetal bovine serum. The cells were collected by centrifugation, washed, and resuspended at 1 x 10^7 cells/ml in complete medium. Bone marrow cells were collected from femurs. After the bones were obtained, skin and muscle were removed. Bone marrow was exposed by cutting the ends of the bones and expelled by inserting a 23 g needle and forcing complete medium through the bone shaft. The cells were collected by centrifugation. After washing, aliquots of 1 x 10^7 cells were diluted in 1 ml of complete medium. To obtain blood samples for NK cell analysis, mice were anesthetized with methoxyflurane and bled retroorbitally. Peripheral blood mononuclear cells, collected from heparinized blood, were isolated by centrifugation over a Histopaque-1077 solution (Sigma-Aldrich, Inc. Atlanta, GA, USA) at 400 x g at a density of less than 1.077 g/cm^3 and at room temperature for 30 min. These cells were then collected at the interface and washed three times in phosphate buffered saline solution. Subsequently, the cell pellet was resuspended with complete medium and adjusted to 1 x 10^7 cells/ml.

Flow cytometry. Flow cytometric analysis was used to evaluate changes in the percentages of NK cells derived from various lymphoid tissues following EtOH administration *in vivo*. Cell suspensions were diluted to 1 x 10^6 cells/100 µl with flow cytometry buffer (phosphate buffered saline with 0.1% bovine serum albumin and 0.1% sodium azide, pH 7.4) and placed in 96-well U bottom culture plates. The rat monoclonal antibody 2.4G2 (Catalog No. 553141, BD Pharmingen, San Jose, CA, USA) was initially added to all

wells (1 μg/well) to block antibody binding via FcμΥreceptors. The plates were kept on ice during the labeling and washing steps. NK cells were identified using the monoclonal antibody PK136 (NK1.1) directly conjugated with phycoerythrin at a concentration of 1 μg/well (Catalog No. 553165, BD Pharmingen, San Jose, CA, USA). Finally, cells were fixed with 200 μl of a 1% paraformaldehyde solution, washed again, and suspended in 200 μl of flow cytometry buffer. Samples were analyzed using a Becton-Dickinson FacsCalibur flow cytometer with 20,000 events for each sample. To detect apoptosis, 5 x 10^5 purified splenic NK cells were fixed in 80% ethanol, then washed with PBS, incubated with 100 μg/ml RNase at 37°C for 30 min, stained with propidium iodide (50 μg/ml), and analyzed on a FacsCalibur flow cytometer. The percentages of apoptotic cells in sub-G1 of the cell cycle were analyzed using Cell-FIT software (Becton Dickinson Instruments, San Diego, CA, USA).

Splenic NK cell purification. NK cells were enriched from mouse spleen using EasySep™ Mouse NK Cell Isolation Kit (StemCell Technologies, Vancouver, British Columbia, Canada) according to the manufacturer's recommendations. Briefly, disrupted spleen was placed in Hanks' Balanced Salt Solution (HBSS) containing 2% fetal bovine serum. Aggregates and debris were removed by passing cell suspension through a 70 μm mesh nylon strainer, followed by centrifugation at 300 x g for 10 min and resuspension at 1 x 10^8 nucleated cells/mL in EasySep™ buffer. Then, 50 μl/ml of Isolation Cocktail were added to the sample for 10 min, followed by the addition of 100 μl/ml of RapidSpheres™ for 5 min. The tube was placed into the StemSep™ magnet and incubated at room temperature for 5 min. Finally, the tube was removed from the magnet with pouring off of the enriched cell suspension for experiments. The enriched NK cell suspensions were assessed by flow cytometry with PK136 antibody staining.

Administration of compounds. Anhydrous absolute EtOH (Millopore, Darmstadt, Germany) was diluted with sterile tissue culture grade water to produce a 32% (v/v) solution. This solution was administered by gavage with an 18-gauge stainless steel gavage needle. Previous studies in this laboratory have shown that administration of EtOH at this concentration does not typically produce histopathological changes in the stomach or duodenum [9]. Vehicle control mice received water only, at the same volume as that of the group receiving EtOH in each experiment. RU486 (Sigma-Aldrich, Inc. Atlanta, GA, USA) was administered by gavage as an aqueous suspension with 0.5% methylcellulose and 0.1% Tween 80 [21].

Western blotting. Cells were lysed in a buffer containing 50mM Tris (pH 7.4), 150mM NaCl, 2mM EDTA, 1mM Na3VO4, 10 mM NaF, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton-100. Equal amounts of proteins (20 μg) were subjected to gel electrophoresis on a 10% gel and then transferred to polyvinylidene difluoride (PVDF) membranes (Pall, Pensacola, FL, USA) by electroblotting. Blots were incubated in a Tris-buffered saline solution at pH7.6, containing 5% nonfat dry milk and 0.1% (v/v) Tween 20. These membranes were incubated at 4°C with primary antibodies (1:1000 dilution) overnight. They were then washed with PBS-Tween 20 and incubated with the appropriate secondary antibodies (1:2000 dilution). An enhanced chemiluminescence kit (Amersham, Arlington Heights, IL, USA) was used to detect the target proteins. Antibodies used in Western blotting were purchased from Santa Cruz Biotechnology (Bax, Catalog No. sc-7480; Bak, Catalog No. sc-832; TNF-R, Catalog No. sc-8436; Fas, Catalog No. sc-1023; Caspase-3, Catalog No. sc-7272; Caspase-8, Catalog No. sc-5263; Caspase-9, Catalog No. sc-133109; β-actin, Catalog No. sc-47778; Santa Cruz, CA, USA). The bands were quantified with densitometric program Image J and the test proteins were divided by β-actin and then normalized by the vehicle control protein.

Statistical analysis. Analysis of Variance (ANOVA) and Dunnett's test were used to evaluate the differences between the vehicle control group and the other groups. (P values determined by Dunnett's test are indicated in the figures.) Student *t*-test was used to determine significant differences between two sets of data. Relevant results are noted in the text. Values of $p < 0.05$ were regarded as

Table 1. Effects of EtOH on NK cell percentages derived from lymphoid tissues
Mice were sacrificed 12 hr after a single dose of EtOH (6.0 g/kg) administered by gavage. Cells derived from spleen, peripheral blood, bone marrow, and peritoneal cavity were prepared for flow cytometric analysis. Values (NK cell percentages) shown are means ± SE of two independent experiments (n = 5 mice per group in each experiment). Values significantly differ between the EtOH and vehicle group on Student's *t* test followed by two-tailed *P* test, as indicated by ** ($p < 0.01$).

NK cells %	*Spleen*	*Peripheral blood*	*Bone marrow*	*Peritoneal cavity*
Vehicle	5.08±0.33	8.55±0.76	2.24±0.15	2.18±0.16
EtOH	3.89±0.21**	6.74±0.57**	1.74±0.02**	1.65±0.02**

significant. All analyses were performed using the Instat software package (GraphPad Software, San Diego, CA, USA).

Results

Effects of EtOH on NK cell percentages derived from lymphoid tissues.

Our previous results demonstrated that EtOH-induced decreases in splenic NK cell percentages are mediated by glucocorticoids [10]. Mice were treated with EtOH at a dosage of 6.0 g/kg for 12 hr. Cell suspensions were collected from lymphoid tissues and NK cell percentages were assessed by flow cytometry. The fate of the lost splenic NK cells was unknown. Glucocorticoids have been shown to alter leukocyte trafficking [11-13]. It is possible that NK cell numbers in the blood or tissues other than the spleen increase following EtOH administration. To determine if the effect of EtOH on decreasing NK cell number was specific to the spleen or more general to lymphoid compartments, NK cell percentages derived from cells of lymphoid tissues were measured. The results indicated that EtOH not only decreases splenic NK cell percentages, but also suppresses NK cell percentages among peripheral blood-, bone marrow- and peritoneal cavity-derived cells (Table 1).

Splenic NK cell purification.

Enriched NK cell suspension obtained with mouse NK cell isolation kit was used to perform flow cytometric analysis to determine percentages of NK cells. Splenic NK cell purity was >90 % as assessed on NK1.1 antibody staining (Figure 1). This result was used to verify NK cell purity for the following experiments.

Time course of EtOH effects on splenic NK cell apoptosis.

To investigate if the loss of splenic NK cells was caused by apoptosis induced by EtOH, purified splenic NK cells were stained with propidium iodide to determine apoptotic percentage. Increase in NK cell apoptosis began 3 hr after EtOH administration and peaked at 6 hr after EtOH administration (Figure 2). The apoptotic percentage returned to a level not significantly different from that of the vehicle control 12 hr after EtOH administration.

Involvement of intrinsic apoptotic pathway in EtOH-induced apoptosis.

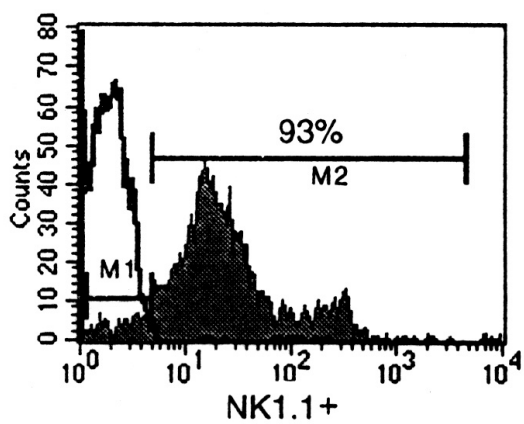

Figure 1. Purity assessment of splenic NK cells with flow cytometric analysis
Splenic NK cells were purified with mouse NK cell isolation kit and labeled with PK136 antibodies for flow cytometric analysis. Cells were assessed as >90 % pure on flow cytometry.

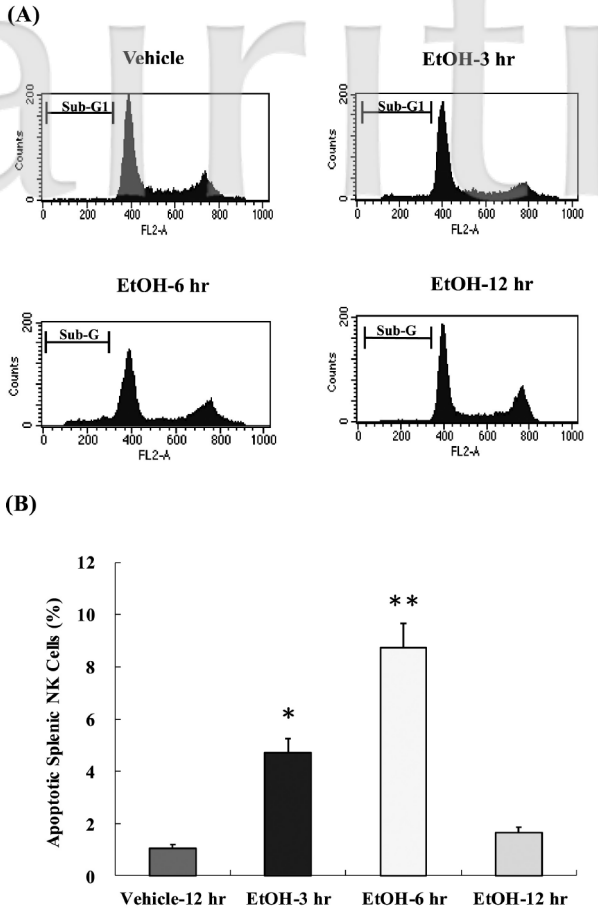

Figure 2. Time course of EtOH effects on splenic NK cell apoptosis
Spleens were removed at the indicated time-points after administration of one dose of EtOH (6.0 g/kg) by gavage. Splenic NK cells were purified with mouse NK cell isolation kit. (A) Apoptotic cells were measured by flow cytometry. (B) Quantified results of apoptotic percentage. Values shown are means ± SE (n = 5 mice per group). Values significantly different from the vehicle control were determined using ANOVA followed by Dunnett's test and indicated by * ($p < 0.05$) or ** ($p < 0.01$).

Spleens were removed and NK cell protein extracts were prepared at the indicated time-points after administration of one dose of EtOH (6.0 g/kg) by gavage. Protein expressions of Bax and Bak slightly increased 3 hr after EtOH administration, reaching a peak at 6 hr after EtOH administration (Figure 3A). TNF-R (TNF-receptor) and Fas protein expressions were not affected by EtOH. TNF-R and Fas activation are involved in the

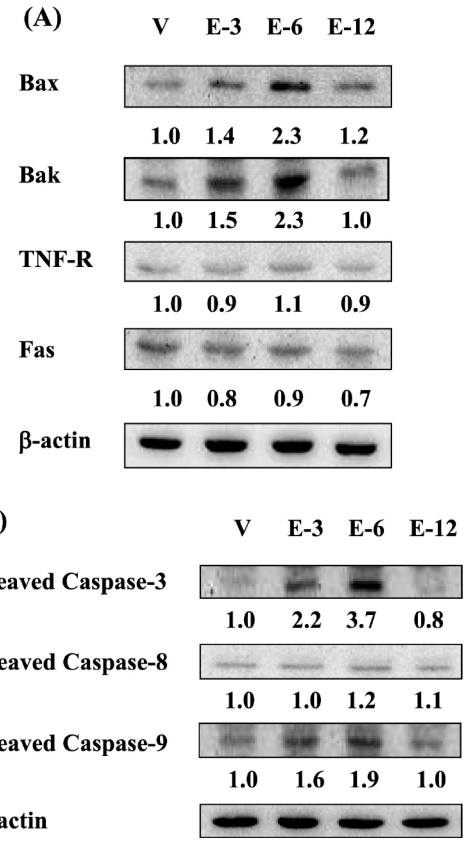

Figure 3. Involvement of intrinsic apoptotic pathway in EtOH-induced apoptosis
Spleens were removed and NK cell protein extracts were prepared at the indicated time-points after administration of one dose of EtOH (6.0 g/kg) by gavage. Splenic NK cells were purified with mouse NK cell isolation kit. The levels of several apoptosis-related proteins (A) and cleaved caspases (B) were determined by Western blotting. Data are expressed as fold change relative to vehicle group.

extrinsic apoptotic pathway. The cleaved forms of caspase-3 and caspase-9 were enhanced by EtOH at 3 hr, reaching a peak at 6 hr. However, the level of caspase-8 did not change following EtOH administration (Figure 3B). These results indicated that EtOH induces splenic NK cell apoptosis via intrinsic apoptotic pathway.

Effect of RU486 on splenic NK cell apoptosis 6 hr after EtOH administration.

RU486 (mifepristone) is a potent glucocorticoid antagonist. It was administered at 200 mg/kg to

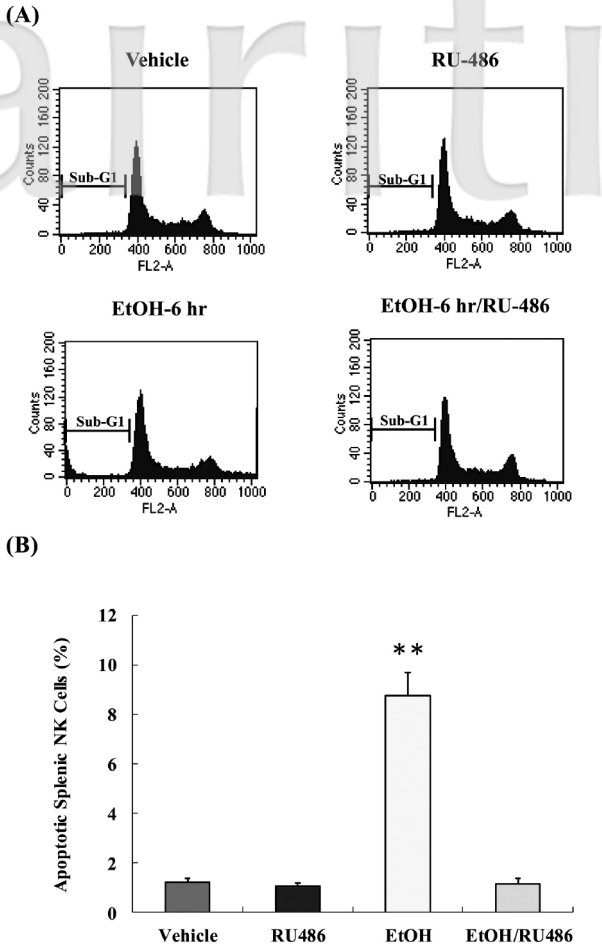

Figure 4. Effect of RU486 on splenic NK cell apoptosis 6 hr after EtOH administration
RU486 (200 mg/kg) was administered by gavage 1 hour before administration of EtOH (6.0 g/kg). Mice were sacrificed 6 hr after a single dose of EtOH (6.0 g/kg) administered by gavage. Splenic NK cells were purified with mouse NK cell isolation kit. (A) Apoptotic cells were measured by flow cytometry. (B) Quantified results of apoptotic percentage. Values shown represent means ± SE from two independent experiments (n = 5 mice per group in each experiment). Values significantly different from the vehicle control were determined using ANOVA followed by Dunnett's test and indicated by ** ($p < 0.01$).

mice 1 hr prior to EtOH administration. Spleens were removed 6 hr after EtOH administration, as at this time-point EtOH administration had the greatest effect on splenic NK cells. Enriched splenic NK cells were prepared for flow cytometric analysis. RU486

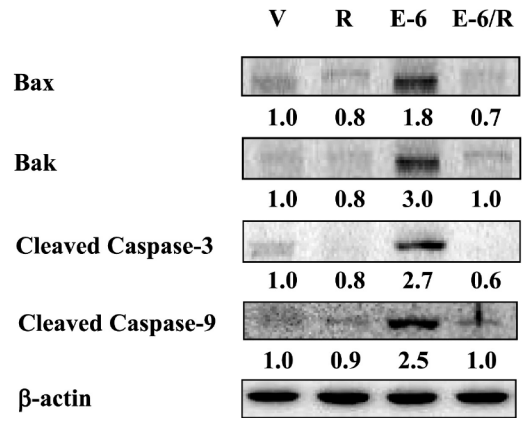

Figure 5. Glucocorticoids are involved in EtOH-induced apoptosis of splenic NK cells
RU486 (200 mg/kg) was administered by gavage 1 hr before administration of EtOH (6.0 g/kg). Mice were sacrificed and protein extracts were prepared 6 hr after a single dose of EtOH (6.0 g/kg) administered by gavage. The levels of apoptosis-related proteins and cleaved caspases were determined by Western blotting.
Data are expressed as fold change relative to vehicle group. V represents vehicle control group, E-6/R denotes mice treated with RU486 and EtOH for 6 hr.

completely blocked the apoptotic effect of EtOH (Figure 4), suggesting the involvement of endogenous glucocorticoids in splenic NK cell apoptosis. RU486 alone had no effect on splenic NK cell apoptosis, as shown in the control group (Figure 4).

Glucocorticoids are involved in EtOH-induced apoptosis of splenic NK cells.

RU486 (200 mg/kg) was administered by gavage 1 hr before administration of EtOH (6.0 g/kg). Splenic NK cell protein extracts were prepared for Western blotting 6 hr after a single dose of EtOH (6.0 g/kg) administered by gavage. RU486 totally blocked the increased expressions of Bax, Bak, cleaved caspase-3, and cleaved caspase-9 induced by EtOH (Figure 5). Taken together with the results of Figure 5 and Figure 6, EtOH may induce endogenous glucocorticoid production and trigger apoptosis of splenic NK cells.

Discussion

Our previous results demonstrated that EtOH-

induced decreases in splenic NK cell percentages are mediated by glucocorticoids [10]. Glucocorticoids are known to alter lymphocyte trafficking [11-13] and it is possible that NK cell numbers in the blood or sites other than the spleen increase following EtOH administration. However, the results from Table 1 indicate a substantial decrease in splenic NK cell numbers in EtOH-treated mice. Moreover, a global suppressive effect of EtOH on NK cell numbers in all tissues and organs tested was observed. These results suggested that the decreased splenic NK cell numbers in EtOH-treated mice are not caused by migration of splenic NK cells to other tested sites.

To investigate if the loss of splenic NK cells is due to apoptosis induced by EtOH, purified splenic NK cells were collected for apoptotic assay. EtOH-induced apoptosis of splenic NK cells reached a peak at 6 hr after EtOH administration (Figure 2). However, the apoptotic percentage returned to normal 12 hr after EtOH administration. This points to apoptosis of splenic NK cells early after EtOH administration. As Bax, Bak, caspase-9, and caspase-3 are major components of the intrinsic apoptotic pathway, these protein expressions were enhanced early after EtOH administration (Figure. 3). These results imply that the intrinsic pathway is involved in the apoptosis of splenic NK cells. Whether the NK cells derived from other lymphoid tissues undergo apoptosis induced by EtOH requires further study.

The results of this study indicated that the chemical stressor EtOH induces apoptosis of splenic NK cells in mice, which is completely blocked by RU486 (Figure 4), suggesting the involvement of glucocorticoids. It has been shown that EtOH at 6.0 g/kg induces a substantial stress response in mice with ~10-fold increases in corticosterone levels [9]. This dosage of EtOH was used in this study. It has been reported that NK cells undergo apoptosis in response to glucocorticoids [14-16]. The loss of splenic NK cells may be due to glucocorticoid-induced apoptosis, as a result of an EtOH-induced stress response.

Glucocorticoid signaling increases the expressions of pro-apoptotic proteins Bax/Bak to disrupt mitochondrial membrane potential, resulting in the release of cytochrome c and other apoptogenic proteins [22]. This leads to caspase 9 activation and subsequent effector caspase 3 activation and apoptosis [23]. Similar results were obtained in this study (Figure 4 and Figure 5). RU486 is a glucocorticoid and progesterone antagonist. However, the results of a study by Weiss et al. suggested that the effects of RU486 in this system are not due to its antiprogesterone activity [24]. It has been noted that acute EtOH exposure decreases progesterone levels [24]. Our previous results demonstrated that RU486 blocks EtOH-induced decreases in splenic NK cell activity [10]. Similar results have been obtained for both male and female mice. Thus, EtOH-induced increases in progesterone levels are not a likely explanation for the apoptotic effects of EtOH.

The purpose of the present study was to assess the role of glucocorticoids in EtOH-induced apoptosis of splenic NK cells. The results indicated that glucocorticoids are involved in the apoptosis of splenic NK cells in binge drinking model and the intrinsic pathway is involved in splenic NK cell apoptosis.

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