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Chronic Ethanol Inhibits NK Cell Cytolytic Activity: Role of Opioid Peptide β-Endorphin

Nadka Boyadjieva,* Madhavi Dokur,* Juan P. Advis,* Gary G. Meadows, † and Dipak K. Sarkar*‡

The role of β-endorphin (β-EP) in ethanol-altered NK cell cytolytic activity is studied using male Fischer-344 rats as an animal model. Ethanol was administered for 1, 2, 3, or 4 wk in a liquid diet containing 8.7% ethanol (v/v), which means that 37% of the total calories were derived from ethanol. Rats treated with ethanol for 1 wk showed an increase in hypothalamic and plasma levels of immunoreactive (IR)-β-EP, but displayed no significant effect on NK cell activity determined by βCr release assay, as compared with those in pair-fed and ad libitum-fed animals. However, animals treated with ethanol for 2, 3, or 4 wk showed decreased hypothalamic and plasma levels of IR-β-EP and decreased splenic NK cell activity. No significant decrease in the number of splenocytes and NK cells or in the percentage of NK cells was seen until after 3 and 4 wk of ethanol treatment. Exposure in vitro of splenic lymphocytes obtained from control animals to various concentrations of β-EP increased NK cell activity. The opioid antagonist naltrexone blocked the β-EP-stimulated effect. The in vitro NK cell response to β-EP was reduced in the splenocytes obtained from animals treated with ethanol for 2 wk, but not in those obtained from animals treated with ethanol for 1 wk as compared with those in control animals. Additionally, β-EP administration into the paraventricular nucleus of the hypothalamus stimulated NK cell cytolytic activity, whereas the opioid blocker administration reduced NK cell activity. The NK cell responses to paraventricular nucleus β-EP were reduced in the animals treated with ethanol for 2 wk. These data provide evidence for the first time that ethanol inhibits NK cell cytolytic activity, possibly by reducing β-EP-regulated splenic NK cell function. The Journal of Immunology, 2001, 167: 5645–5652.

N
atural killer cells have been implicated in immune surveillance against tumors and bacterial and viral infections (1–3). Activated NK cells circulate through blood and lymph and accumulate at sites of injury, infections, and tumors (4). Although an increased incidence of certain forms of cancers and infections has been documented in chronic alcoholics, very few studies have been conducted on the NK cell functions. Although some inconsistencies in findings exist, most studies indicate that NK cell cytotoxicity is reduced in ethanol-treated animals (5–10). A few studies conducted using humans also show reduction in NK cell functions following alcohol administration (11–13). The mechanism by which ethanol alters NK cell function is not known.

NK cells quickly respond to immune activation signals (14–16). NK cell cytotoxic activity is increased by the lymphokine IFN-γ, which has a number of opioid-like effects (17). Additionally, IFN-γ-mediated NK cell cytolytic activity is blocked by the opioid receptor antagonist (18). The opioid peptide β-endorphin (β-EP) is produced in the hypothalamus, in the pituitary, and in other peripheral glands (19–21). β-EP enhances splenic lymphocyte proliferation in response to concanavalin A and increases the killing activity of NK cells (22–24). Opioid receptors are present on lymphocytes (25). Because the function of endogenous β-EP is altered in alcoholic patients and alcohol-treated animals (26–29), questions arise as to whether the abnormality in immune function, particularly the changes in NK cell activity in the alcoholic, is secondary to altered β-EP function. The present study is conducted to investigate the role of β-EP in ethanol-modulated NK cell functions using the Fischer-344 male rat as an animal model. In this report, we provide evidence that ethanol-induced inhibition of NK cell cytolytic activity may be partly due to reduced β-EP-regulated NK cell function.

Materials and Methods

Animals and feeding design

Male Fischer-344 rats of 150–175 g body weight were maintained on a 12-h light/dark cycle (lights on 7:00 a.m. and lights off at 7:00 p.m.) and were either ad libitum-fed rodent chow, pair-fed an isocaloric liquid diet, or fed an ethanol-containing liquid diet as described by us previously (30). The liquid diet consisted of vitamins (0.31%, w/v), minerals (0.50%, w/v), sucrose (11.36%, w/v; pair-fed group), or ethanol (8.7%, v/v; ethanol-fed group) and Sustacal (83.3%, v/v; Mead Johnson, Evansville, IN). Ethanol substitution for sucrose provided ~37% ethanol-derived calories to the ethanol-fed rats. Graduated ball-barrel cylinders containing the freshly prepared diet were placed in the animals’ cages 1 h before lights off (6:00 p.m.) for 1, 2, 3, or 4 wk. Animals were given free access to water. The body weights of the animals were recorded before sacrifice by decapitation. At the end of experiments, animals were weighed and decapitated. Spleen tissues were immediately removed and used for NK cell cytolytic activity. The hypothalamic tissues and trunk blood samples were also taken for analysis of tissue and plasma immunoreactive (IR)-β-EP levels. Animal surgery and care were in accordance with institutional guidelines and complied with National Institutes of Health policy.
**Intrarparaventricular administration of β-EP or naltrexone**

Male Fischer rats weighing 150–175 g body weight were anesthetized with sodium pentobarbital (33 mg/kg body weight) and were stereotactically implanted with a bilateral guide cannula (Plastics One, Ronkonkoma, NY) using a Kopf stereotaxic apparatus. In each rat, bilateral guide cannulae were positioned 3 mm apart, immediately lateral to each paraventricular nucleus (PVN; 1.8 mm behind bregma, 1.5 mm lateral to the midline, and 7.7 mm below the skull surface) and were closed by a dummy infusion cannula. Seven days after stereotaxic surgery, rats were pair-fed or ethanol-fed for an additional 2 wk. At the end of this period, β-EP, naltrexone, or artificial cerebrospinal fluid (CSF) was infused into each PVN. The β-EP and naltrexone solutions were made using artificial CSF, and the concentration of these solutions was 200 ng/μl. Five microliters of β-EP or naltrexone or CSF were infused through each PVN cannula during a 5-min period. The animals that received β-EP were additionally infused with 100 ng of β-EP/0.5 μl/h in each PVN for a period of 16 h. Control animals received an additional 0.5 μl CSF/h in each PVN for a period of 16 h. β-EP or CSF was continuously perfused using an Alzet mini pump (model 2002; ALZA, Palo Alto, CA). The osmotic pump was implanted subcutaneously 4 h after infusion to prevent cannula occlusion by CSF. All surgery was done under isoflurane anesthesia. Each infusion cannula reached the PVN through the permanently implanted bilateral guide cannula. Rats infused with naltrexone were sacrificed 4 h after treatment, whereas β-EP- or CSF-treated animals were sacrificed after completion of the 16-h infusion. Immediately after sacrifice, spleen tissues and brains were removed. Spleens were used for NK cell cytolytic activity. Brains were frozen on dry ice until brain sequential coronal sections were performed to verify the exact position of the infusion site.

**Flow cytometric analysis**

Flow cytometric analysis was used to determine the percentage of NK cells in spleen cell preparations. Splenic lymphocytes were isolated as described above in medium containing RPMI 1640, 1% penicillin and streptomycin, 2% glutamine, and 10% untreated FBS and were pelleted by centrifugation. Cells were resuspended in flow cytometry buffer (PBS with 0.1% BSA and 0.1% sodium azide, pH 7.4) at a concentration of 10^6 cells/100 μl. Each sample was washed twice, and finally suspended in 200 μl of flow cytometry buffer for analysis. Flow cytometric analysis was performed on a FACScan with Lysis 2 data analysis software (BD Biosciences, Mountain View, CA). Lymphocytes were gated according to forward and side scatter properties. Data were accumulated for 10^6 gated cells.

**NK cell activity**

NK cell cytolytic activity of splenic lymphocytes was determined against yeast artificial chromosome (YAC)-1 lymphoma cells by a standard 4-h 51Cr release cytolytic assay as described by us previously (2). Splenocytes were obtained from whole spleens by pressing tissue through stainless steel wire-mesh screens. Erythrocytes were removed by a 5-s hypotonic shock with sterile distilled water. The percentages of cytolytic activity at E:T ratios of 200:1, 100:1, 50:1, and 25:1 were converted to lytic units per 10^6 effector cells according to Pross et al. (31). Each assay was conducted in quadruplicate.

**Table I. Changes in body weight at different time intervals in animals fed with a rodent chow ad libitum, fed with a liquid ethanol diet (alcohol-fed), or pair-fed a liquid diet with sucrose substituted for ethanol (pair-fed) for 4 wk**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0 Day</th>
<th>5 Days</th>
<th>10 Days</th>
<th>21 Days</th>
<th>28 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad lib-fed</td>
<td>145 ± 2</td>
<td>168 ± 3</td>
<td>195 ± 6</td>
<td>220 ± 4</td>
<td>232 ± 4</td>
</tr>
<tr>
<td>Pair-fed</td>
<td>150 ± 4</td>
<td>170 ± 6</td>
<td>190 ± 5</td>
<td>212 ± 3</td>
<td>227 ± 3</td>
</tr>
<tr>
<td>Alcohol-fed</td>
<td>140 ± 3</td>
<td>161 ± 3</td>
<td>185 ± 2</td>
<td>208 ± 4</td>
<td>217 ± 3*</td>
</tr>
</tbody>
</table>

*a Data are mean ± SE of five animals.

*, p < 0.001 significantly different from the ad libitum-fed and pair-fed groups at the same time period.

**Assay for blood levels of ethanol**

Ethanol levels in plasma samples were determined using a commercially available enzymatic assay (Sigma-Aldrich, St. Louis, MO), where ethanol concentrations were determined from the absorbency at 340 nm. The minimum amount of alcohol level detected by this assay was 10 mg/dl.

**Radioimmunoassay (RIA) of IR-β-EP**

The IR-β-EP levels in plasma and the hypothalamus were measured by a RIA as described by us previously (32). Hypothalamic tissues were extracted with 0.1 N HCl, and 10 μl of extract of each sample was used in duplicate in the RIA. Plasma samples from blood were obtained and used directly for measurement of IR-β-EP levels. The RIA system used a β-EP antiserum Y10 (S. S. C. Yen, University of California, San Diego, CA), which cross-reacted with β-EP (100%) and with β-lipotropin (15–20%) on a molar basis. The minimum amount of β-EP detectable was 3 pg/tube at 1/28,000 antiserum dilution. IR-β-EP characterized by gel chromatography from hypothalamic tissue extracts and from plasma extracts showed a major β-EP component and a small β-lipotropin component (32), suggesting that the values obtained by this RIA represented mostly β-EP peptides. The protein content in hypothalamic extracts was determined using the bicinchoninic acid (Pierce, Rockford, IL) protein assay reagents. Protein values were used to calculate the amount of IR-β-EP per microgram of protein in the tissue samples.

**Statistics**

The mean and SE of the data were determined and are presented in the text and figures. Data were analyzed using one-way ANOVA. The differences between groups were determined using the Student-Newmann-Keuls test. A value of p < 0.05 was considered to be a significant difference.

**Results**

**Blood alcohol levels and body weights**

As expected from our previous findings (30), the alcohol treatment regimen significantly elevated the levels of blood alcohol (n = 5; milligrams per deciliter; day 7, 93.7 ± 16.0; day 14, 131.4 ± 21.0; day 21, 127.1 ± 9.0; and day 28, 119.0 ± 7.6). The liquid diet regimen used for alcohol administration did not produce a nutritional deficit because body weights of pair-fed and ad libitum-fed rats were not significantly different. However, alcohol-fed rats showed significantly (p < 0.01) reduced body weight gain as compared with pair-fed and ad libitum-fed rats after 4 wk of treatment (Table I).

**Basal NK cell cytolytic activity**

The effect of ethanol consumption on splenic NK cell activity was determined in rats given an ethanol-containing diet for 1, 2, 3, and 4 wk. As shown in Fig. 1, ethanol intake for 2, 3, and 4 wk reduced NK cell activity as compared with the activity of pair-fed or ad libitum-fed animals. Ethanol intake for 1 wk did not have any significant effect on NK cytolytic activity. The cytolytic activities of 1-wk ethanol-treated animals were similar to those of pair-fed or ad libitum-fed animals.
cell function. To test this possibility, we determined the effects of an involvement of the opioid peptide in alcohol-regulated NK levels of IR-EP. The parallel changes in NK cell activity and tissue and plasma antagonist naltrexone reduced levels of IR-EP in the hypothalamic extracts. As shown in Fig. 2A, animals given an ethanol diet for 2, 3, or 4 wk had significantly reduced levels of IR-EP in the hypothalamic extracts as compared with those in pair-fed or ad libitum-fed animals. In contrast, animals given the ethanol diet for 1 wk had significantly increased levels of IR-EP in the hypothalamic extracts. Similarly, the plasma levels of IR-EP were significantly higher following 1 wk of ethanol consumption, but were significantly reduced after 2, 3, or 4 wk of ethanol consumption (Fig. 2B).

Effects of chronic ethanol on NK cell numbers in the spleen

Flow cytometric analysis was used to determine the percentage of NK cells in spleen cell preparations. As shown in Fig. 3, a–c, animals given an ethanol diet for 1 and 2 wk had no significant change in splenocyte number, total NK cell number, or percentage of NK cell population. However, animals given an ethanol diet for 3 and 4 wk had significantly reduced number of splenocytes, total NK cell population, and percentage NK cell population.

NK cell cytolytic response to in vitro β-EP and opiate antagonist naltrexone

The parallel changes in NK cell activity and tissue and plasma levels of β-EP following alcohol treatment suggest the possibility of an involvement of the opioid peptide in alcohol-regulated NK cell function. To test this possibility, we determined the effects of β-EP and the opioid receptor blocker naltrexone on splenic NK cell activity in vitro. As shown in Fig. 4A, β-EP increased the in vitro NK cell cytolytic activity of splenocytes in a concentration-dependent manner. Naltrexone inhibited the β-EP-stimulated NK cell activity (Fig. 4B). Naltrexone by itself did not have any effect on in vitro NK cell activity. As shown in Fig. 5, 1 wk of ethanol consumption did not affect the in vitro NK cell response to β-EP. However, 2 wk of ethanol consumption significantly reduced the in vitro NK cell cytolytic response to β-EP.

NK cell cytolytic response to in vivo β-EP and opiate antagonist naltrexone

The role of β-EP in ethanol-modulated NK cell function was evaluated in vivo by determining the effects of hypothalamic administration of β-EP or naltrexone on splenic NK cell activity. The opioid peptide and its blocker were infused into the PVN, as a large number of β-EP terminals and β-EP-sensitive receptors were localized in this part of the hypothalamus (19, 33–35). As shown in Fig. 6, β-EP infusion into the PVN increased the NK cell cytolytic activity in ad libitum- or pair-fed control animals. The infusion of the opioid peptide into the PVN failed to increase NK cell activity in animals fed with an alcohol diet for 2 wk. However, naltrexone failed to significantly reduce NK cell activity in animals fed with alcohol for 2 wk.
The data obtained from in vivo studies presented in this study demonstrate that ethanol consumption for 2, 3, or 4 wk suppresses NK cell activity and that ethanol’s inhibition of NK activity is associated with decreased plasma and hypothalamic content of β-EP in male rats. In vivo and in vitro studies also provided evidence that central β-EP and peripheral β-EP increase NK cell activity and that an opioid receptor antagonist, naltrexone, blocks these responses. Additionally, ethanol-treated animals showed a reduced NK cell response to β-EP. Together, these data provide evidence for the first time that chronic administration of alcohol reduces NK cell cytolytic activity by altering β-EP-regulated NK cell function.

Discussion

The data obtained from in vivo studies presented in this study demonstrate that ethanol consumption for 2, 3, or 4 wk suppresses NK cell activity and that ethanol’s inhibition of NK activity is associated with decreased plasma and hypothalamic content of β-EP in male rats. In vivo and in vitro studies also provided evidence that central β-EP and peripheral β-EP increase NK cell activity and that an opioid receptor antagonist, naltrexone, blocks these responses. Additionally, ethanol-treated animals showed a reduced NK cell response to β-EP. Together, these data provide evidence for the first time that chronic administration of alcohol reduces NK cell cytolytic activity by altering β-EP-regulated NK cell function.
The action of ethanol on NK cells in rats has not been well studied. To our knowledge, this is the first study in which time-dependent effects of ethanol on NK cells were determined. With careful attention to nutritional status and ethanol intake, studies in which mice are given high levels of ethanol (20%, w/v) in the drinking water showed inhibition of NK cell cytolytic activity (2, 6, 7). Wu and Pruett (8, 10), in a binge alcohol model, also showed inhibition of NK cell cytolytic activity and a decrease in cell percentage and number in mice. Data presented in this report indicate that ethanol has an inhibitory effect on NK cell cytolytic activity independent of NK cell loss at 2 wk. Thereafter, it is probably a combination of loss and the independent effect on NK cytolytic activity.

The relevance of rodent models of alcohol intake to NK cells in humans modulated by alcohol is still largely unknown. Assessments of NK cell activity in humans is complicated by the fact that many patients use tobacco and other drugs that are known to affect NK cell activity (36, 37). Furthermore, alcoholics are frequently malnourished (38). They may have underlying viral infections and may have varying degrees of liver damage, which also affects immune responses. Patients with chronic active hepatitis and cirrhosis have low NK activity (39, 40). However, it is unclear whether the immunological abnormalities in human alcoholics result from generalized malnutrition, malabsorption of specific nutrients, or direct ethanol exposure (38, 41, 42). Charpentier et al. (43) found that NK cell activity was impaired only in alcoholic subjects with inactive cirrhosis. Additionally, the decrease was more pronounced in cirrhotic patients with severe malnutrition. In contrast, Saxena et al. (44) observed enhanced NK cell activity in peripheral blood lymphocytes from alcoholic subjects despite compounding factors such as inadequate nutritional status, clinical illness, drug addiction, and the fact that 70% of the subjects smoked. In other studies, sober chronic alcoholics who were well nourished and free of underlying diseases had no demonstrable abnormality in immune function, including NK cell activity (45). Additionally, NK cell activity was decreased in depressed patients with alcoholism (12). A recent publication by Cook et al. (13) showed a trend...
toward lower NK cell activity in alcoholics without liver disease and a loss of NK cells in alcoholics with liver disease. Although additional research is needed to clarify the role of alcohol in human alcoholics, a majority of these studies suggest a reduced NK cell function.

The data on the varying effects of ethanol on hypothalamic β-EP are in agreement with previously published studies in rats (26–29). It has been shown that acute treatment of ethanol stimulates hypothalamic content of β-EP and its precursor proopiomelanocortin (POMC) mRNA, whereas chronic treatment with ethanol reduced β-EP and POMC mRNA levels in this tissue. Similar biphasic actions of ethanol on β-EP and POMC mRNA levels were observed in hypothalamic cells in primary culture (46–49). It is believed that hypothalamic β-EP neurons, when exposed to a longer duration of ethanol, develop desensitization due to adaptive changes in cellular signaling mechanisms (50). The data presented in this study suggest that the plasma β-EP exhibits a biphasic response to ethanol depending on the length of treatment. The source of β-EP in plasma is believed to be primarily from the pituitary gland (19). However, it is also possible that the other peripheral tissues, including the adrenal gland and lymphocytes, contribute to the circulatory levels of β-EP (20, 21). It could be concluded that ethanol administration for a period of 2 wk or longer also reduces β-EP secretion from the peripheral organs that contribute to the circulatory β-EP.

The results presented in this study demonstrate that the reduction of hypothalamic and plasma β-EP following chronic ethanol intake correlates with the reduction of NK cell cytolytic activity. The positive association between the changes of NK cell function and hypothalamic β-EP levels in ethanol-treated animals provides correlating evidence for an involvement of the opioid peptide in controlling NK cell function. This view is further supported by both in vivo and in vitro data that β-EP stimulates NK cell function, which is blocked by the opiate antagonist naltrexone. The ability of morphine and enkephaline to affect various types of immune cells has been documented previously (51–55). However, there are contradictory evidence from studies on enkephaline and morphine on NK cell functions. The treatment with morphine decreased NK cell activity (53, 54), whereas the treatment with enkephaline increased NK cell activity or had no effect (51, 52). The morphine inhibitory effect on NK cell appears not to be mediated via hypothalamic opioid system, as the morphine effect is only observed after direct administration into the periaqueductal gray matter but not after administration into different areas of the hypothalamus (55).

The stimulatory action of β-EP on NK cell function could occur by increasing the killing activity or by increasing the cytokine secretion. The data presented in this study revealed the cytolytic activity of NK cells. In a preliminary study, we determined the plasma immunoreactive IFN-γ levels in alcohol-treated rats and pair-fed animals. We found no changes in the plasma levels of IFN-γ following 2 and 4 wk of alcohol administration (data not shown). Although these data support the concept that β-EP increases the killing activity of NK cells, further studies determining in vivo and in vitro levels of cytokines (e.g., IFN-γ), killing molecules (e.g., perforin and granzyme), and NK surface markers (e.g., Fas ligand) are needed to establish the mechanism by which the opioid peptide alters NK cell function.

Previously, it has been shown that the β-EP peptide enhances splenic lymphocyte proliferation in response to concanavalin A and increases the killing activity of NK cells (18, 22–24). IR-β-EP is also present in lymphocytes (56) and macrophages (20). POMC mRNA transcript has been identified in splenic lymphocytes (57, 58). Both acetylated and nonacetylated β-EP are produced in splenic lymphocytes (20). [125I]β-EP can bind to splenocytes (26), and the bound [125I]β-EP can be displaced by N-acetylated β-EP and C-terminal fragments of β-EP but not by naloxone (25). Recently, a high-affinity ε-like opioid receptor has been identified on lymphocytes that is specific for β-EP (59). In addition to these high-affinity opioid receptors, lymphocytes possess δ, κ, and μ opioid receptors (59). It has been shown that endogenous opioids up-regulate human and rat NK cell activity (18, 22). Hence, it appears that β-EP is an important regulator of NK cell function.

Although a positive association between β-EP and NK cell activity following 2 wk of ethanol treatment was observed in this study, the early action of ethanol on β-EP and NK cell function did not correlate positively. Considering the stimulatory action of β-EP on NK cells, we anticipated that initial ethanol consumption would stimulate NK cells because ethanol treatment stimulates hypothalamic and peripheral β-EP. However, 1 wk of ethanol treatment did not significantly change NK cell function. One explanation for this discrepancy is that during the early phase of action, ethanol stimulation of β-EP-regulated NK cell function was overcome by NK cell inhibitory factors. It has been shown previously that acute ethanol treatment elevates secretion of hormones from the hypothalamic pituitary adrenal axis, leading to increased release of plasma corticosterone and hypothalamic corticotropin-releasing hormone (60–62). Although the inhibitory influence of glucocorticoids on NK cell function remains to be elucidated in humans (63–65), studies in rats suggest that corticotropin-releasing hormone activates the sympathetic nervous system and releases norepinephrine to inhibit NK cell function (66–68). Hence, it is possible that the stimulatory influence of β-EP during the early ethanol administration is also compensated by the inhibitory influences of the hormones of the hypothalamic pituitary adrenal axis.

In summary, the data presented in this study indicate that β-EP stimulates NK cell cytolytic activity. Additionally, the lymphocytes appear to produce the receptors for the opioid peptide (65). In this study, we have presented evidence that chronic administration of ethanol reduces the levels of both hypothalamic and peripheral β-EP. We have also provided evidence that the influence...
of hypothalamic and peripheral β-EP on NK cell cytolytic activity is reduced by ethanol treatment. This addictive agent lowered the response to VPN-administered β-EP, as well as the direct response to β-EP in vitro. Hence, alcohol’s inhibitory action on NK cell function may involve a reduction in both.

References