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The Role of Stress Mediators in Modulation of Cytokine Production by Ethanol

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Abstract

Acute ethanol exposure in humans and in animal models activates the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS); the resultant increases in concentration of neuroendocrine mediators contribute to some of the immunosuppressive effects of ethanol. However, the role of these mediators in the ethanol-induced inhibition of inflammatory responses is not clear. This is complicated by the fact that most inflammatory stimuli also activate the HPA axis and SNS, and it has not been determined if ethanol plus an inflammatory stimulus increases these stress responses. Addressing this issue is the major focus of the study described herein. Complementary approaches were used, including quantitative assessment of the stress response in mice treated with polyinosinic-polycytidylic acid (poly I:C, as an inflammatory stimulus) and inhibition of the production or action of key HPA axis and SNS mediators. Treatment of mice with ethanol shortly before treatment with poly I:C yielded a significant increase in the corticosterone response as compared to the response to poly I:C alone, but the increase was small and not likely sufficient to account for the anti-inflammatory effects of ethanol. Inhibition of catecholamine and glucocorticoid production by adrenalectomy, and inhibition of catecholamine action with a sustained release antagonist (nadalol) supported this conclusion and revealed that “excess” stress responses associated with ethanol treatment is not the mechanism of suppression of pro-inflammatory cytokine production, but stress-induced corticosterone does regulate production of several of these cytokines, which has not previously been reported.

Keywords

catecholamines; corticosterone; cytokines; ethanol; poly I:C

Introduction

Acute high dose ethanol exposure inhibits innate immunity, increasing susceptibility to infection by a number of pathogens (Nelson and Kolls, 2002). Since acute, or binge drinking,

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is prevalent in the U.S. (Wechsler *et al.*, 2002), insight into the mechanisms by which it dampens inflammatory responses could be useful in developing strategies to prevent the immunosuppression by ethanol and possibly other stressors. We have developed a mouse model for binge drinking to examine these mechanisms (Carson and Pruett, 1996) and have demonstrated that some of these effects are mediated by an ethanol-induced increase in glucocorticoids (corticosterone in mice) via activation of the HPA axis (Han *et al.*, 1993; Han and Pruett, 1995; Weiss *et al.*, 1996; Pruett *et al.*, 2003). The SNS is also activated by acute ethanol exposure, but the role of catecholamines in immunological effects of ethanol has not been evaluated as extensively as the role of glucocorticoids. Results from one study indicate that both catecholamines and corticosterone contribute to suppression of natural killer (NK) cell activity (Wu and Pruett, 1997). Sympathetic nerves innervate lymphoid organs and most immune cells express adrenoreceptors, and it has been suggested that the sympathetic response to each immune challenge may produce different effects locally vs. systemically (Elenkov *et al.*, 2000) *in vivo*. Although most studies in this field have employed cultured primary cells or cell lines, it would seem more appropriate to evaluate these effects using an *in vivo* exposure system.

Inflammatory stimuli have long been known to induce classical neuroendocrine stress responses, and there are indications that glucocorticoids can act as a feedback inhibitor to limit the production of pro-inflammatory cytokines induced by such stimuli (Fantuzzi *et al.*, 1995). This complicates interpretation of previous studies in which we found that abrogating the glucocorticoid response to an inflammatory stimulus (using a glucocorticoid synthesis inhibitor and a glucocorticoid antagonist) did not seem to diminish the inhibition of pro-inflammatory cytokine production by acute administration of ethanol (Pruett *et al.*, 2003b; Glover and Pruett, 2006). Furthermore, a role for the SNS in the ethanol-induced suppression of pro-inflammatory cytokine production or enhancement of IL-10 production was not considered in our previous studies. The study described here was conducted to address these issues. A time-course study was conducted to determine if ethanol given shortly before a potent inflammatory stimulus causes a substantially larger increase in serum corticosterone over time than the inflammatory stimulus alone. In addition, two methods were used to determine if stress-induced catecholamines play a role in the actions of ethanol in this experimental system. Adrenalectomy (to prevent the stress-induced surge in adrenal-derived epinephrine and norepinephrine) and a continuous release form of β -adrenergic antagonist (nadolol) yielded the same fundamental conclusion with regard to the role of adrenergic receptors in the effects of ethanol in this experimental system.

Materials and Methods

Mice

Female B6C3F1 mice (6-8 weeks of age) were purchased through the National Cancer Institute's Animal Program. These mice have been used in this lab for many years due to their extensive use in studies sponsored by the National Toxicology Program. They were selected for use in the present study primarily because this will facilitate comparisons to our previous results and allow use of dosages of poly I:C and other reagents already optimized for this strain. Female mice were used in these studies because group-housed male mice exhibit aggressive behavior that causes unpredictable stress responses. Adrenalectomized (ADX), sham adrenalectomized (sham), and untreated female B6C3F1 mice (6-8 weeks of age) were purchased from Charles River Laboratories for the ADX mice experiment. All mice were allowed to acclimate for 2 weeks and were housed on a 12-hour light/dark cycle in a temperature- and humidity-controlled facility accredited by the American Association for Accreditation of Laboratory Animal Care. Animal care and use was in accord with the NIH

Guide and LSUHSC policies. ADX mice were given 0.9% NaCl solution in place of their drinking water to compensate for lack of mineralocorticoids in ADX mice.

Area under the corticosterone concentration vs. time curve

Groups of mice (5 per group) included at each time point were naive (untreated), poly I:C (Sigma Chemical Co, St. Louis, MO), poly I:C + ethanol (absolute, Pharmco-AAPER, Shelbyville, KY), and ethanol plus the vehicle for poly I:C (phosphate buffered saline, Sigma-Aldrich, St. Louis, MO). Ethanol or vehicle (tissue culture grade water from Sigma-Aldrich), was administered by gavage at a dosage of 6 g/kg using a 32% (v/v) solution in water. Fifteen minutes later, poly I:C or vehicle (phosphate buffered saline) was administered at a dosage of 100 µg/mouse, intravenously in 0.1 ml. Trunk blood was collected by decapitation at 1, 2, 4, 6, 8, and 10 hours after poly I:C dosing. Each cage of 5 mice was bled within 3 minutes, and all unnecessary entry into the animal rooms was avoided to reduce handling related increases in corticosterone levels. Naive groups received no treatments and were left undisturbed until they were bled.

Experiments with adrenalectomized (ADX) mice

Adrenalectomized (ADX), sham ADX, or surgically untreated mice were used in these experiments. These mice were purchased at the same time from the same supplier. Placebo, corticosterone (5.0 mg) and/or nadolol (0.5 mg) pellets (Innovative Research of America, Sarasota, FL) were placed subcutaneously at the back of the neck while mice were under anesthesia with pentobarbital (45 mg/kg, intraperitoneally). The dosage of corticosterone pellet was selected from previous studies with ADX mice (Pruett *et al.*, 2004), and was chosen as the best dose to match or slightly exceed the corticosterone levels induced in a stress response. Sustained release nadolol pellets from Innovative Research of America at the dosage used here have been reported in numerous articles to block sympathetic responses (Barazzone-Argiroffo *et al.*, 2003; Kelley *et al.*, 2002; Ghoshal *et al.*, 1998; Dobbs *et al.*, 1993). Administration of test reagents and collection of samples were performed the day after implantation of pellets. Mice in groups of 4 were left untreated or were treated with ethanol or vehicle (water or 0.9% saline, respectively for normal and ADX mice) 15 minutes prior to treatment with poly I:C (Invivogen, San Diego, CA) or vehicle (PBS). Our previous work indicates that this poly I:C is essentially endotoxin free (Pruett, *et al.*, 2004). Ethanol was administered by oral gavage with a 32% v/v solution in water or in 0.9% saline, at a dosage of 6 g/kg. Poly I:C was administered intravenously at a dosage of 100 µg/mouse in 0.1 ml sterile PBS. Two hours after poly I:C administration, trunk blood was collected by decapitation. This time point was selected as optimum for most cytokines using the data from the AUC experiment (which was essentially a time course experiment).

Measurement of corticosterone, cytokines, and chemokines

Serum corticosterone concentrations were measured using a radioimmunoassay kit (Diagnostic Products Corp., Los Angeles, CA) for determination of AUC values, but due to problems with availability, an ELISA kit (Assay Designs, Ann Arbor, MI) was used subsequently. These kits yielded comparable results when the same samples were evaluated (data not shown). Cytokines and chemokines were measured using a Bioplex multiplex assay kit (Bio Rad, Hercules, CA) and Bioplex reader. Interleukin-6 concentrations were outside the upper range of the standards, so serum samples were diluted and IL-6 was measured using ELISA (BD Biosciences, San Diego, CA). Quantitation of IFN- α was accomplished using an ELISA kit (PBL Interferon Source, Piscataway, NJ).

Calculation of the area under the corticosterone concentration vs. time curve (AUC) and statistical methods

Corticosterone AUC values from 0 to 2 hours and 0 to 8 hours were calculated. The 2 hour time point was chosen because it was the peak time or shortly before the peak time for expression of most cytokines following poly I:C treatment as well as the best time point to demonstrate cytokine alterations by ethanol administration. The corticosterone value for mouse 1 from each treatment group was then plotted up to the chosen time using Prism 4.0 software (GraphPad, San Diego, CA), and the AUC was calculated using the AUC feature in this software. The remaining mice in each treatment group were plotted in the same way, so 5 individual AUC values were obtained for each treatment group. The means and statistical significance between groups were determined using analysis of variance followed by Newman-Keul's post hoc test. Before this analysis, the mean AUC was calculated for the naive group and subtracted from the AUC value for each treatment group. Thus, the AUC values used reflect only the increase in corticosterone exposure over time, not normal circadian changes.

Results

Serum corticosterone AUCs

We began by determining the time course of changes in serum corticosterone concentrations in mice treated with ethanol, poly I:C, or both. As shown in Fig. 1, corticosterone rose sharply in poly I:C-treated mice within the first 1-2 hours and gradually returned to levels observed in untreated mice within 8 hours. Notably, the corticosterone concentrations in ethanol-treated groups increased more rapidly, and corticosterone concentrations in the group treated with poly I:C plus ethanol remained elevated at higher concentrations for a longer period of time than either of the other two treatment groups. Due to these differences, the area under the concentration vs. time curve (AUC) for the poly I:C + ethanol group was greater than either individual treatment when calculated for 2 hours or 8 hours (Fig. 2). However, comparison of the increase in corticosterone AUC with results from our previous studies indicates that a change in corticosterone of the magnitude shown in Fig. 2 would have a very small effect on even the most sensitive immunological parameter evaluated in our previous studies (expression of MHC class II proteins on B lymphocytes) (Pruett *et al.*, 2003c). The percentage of MHC II high expressing B cells would be 15.0% in the group treated with poly I:C alone and 12.3% in the group treated with poly I:C + ethanol (using calculations described previously) (Pruett *et al.*, 2003c). These studies revealed a highly significant linear correlation between MHC class II expression on B cells and area under the corticosterone concentration vs. time curve. It is unlikely that the increased exposure to corticosterone that only causes change of 2.7% in expression of MHC (which is one of the most sensitive immunological end points we have evaluated) could account for the almost complete inhibition of cytokine production observed in the present study. The other approach used in this and previous studies (blocking the action or production of corticosterone and catecholamines), reinforces the idea that the increased glucocorticoids following acute ethanol administration are not a major factor in the immunosuppressive effects of ethanol on cytokine production (Glover and Pruett, 2006).

Serum cytokines in AUC experiment

In the experiment shown in Fig. 3, we examined the effects of ethanol alone or in combination with poly I:C on levels of cytokines and chemokines *in vivo*. Ethanol treatment alone did not cause an increase in any of the cytokines or chemokines tested. As expected, the pro-inflammatory cytokines TNF- α , IL-12 (p40), and IL-12 (p70) were increased by poly I:C treatment, and this increase was at least partially abrogated by ethanol. Of the cytokines tested, the production of IL-1 β , IL-6, and IL-10 were not inhibited by ethanol. Chemokines tested were MIP-1 α , MIP-1 β , and KC. Of these, MIP-1 α and MIP-1 β were not significantly inhibited by ethanol. KC results in both the poly I:C and poly I:C + ethanol groups were above the range

of the standard curve at 1 and 2 hours, later dropping down to naive levels by 8 hours (data not shown). Thus, it is not clear if there was a difference, but a large quantity of KC was produced in both the presence and absence of ethanol.

Effects of adrenalectomy, corticosterone replacement, and beta blocker on corticosterone, cytokine, and chemokine concentrations following treatment with poly I:C with or without ethanol

The strategy in this experiment was to evaluate the role of corticosterone (by adding corticosterone back to ADX mice with sustained release pellets), systemic catecholamines (which would not increase as normal in ADX mice), and peripheral catecholamines (the effects of which on β 2-adrenergic receptors are blocked by nadolol). As expected, ADX mice without corticosterone replacement pellets produced virtually no corticosterone when treated with poly I:C or poly I:C + ethanol, whereas sham ADX, non-ADX mice with nadolol pellets, and ADX mice with corticosterone replacement pellets all had elevated corticosterone levels (Fig. 4A). It should be noted that all corticosterone concentrations here (except those in ADX mice) are greater than the 50-250 ng/ml (depending on time of day) corticosterone concentrations we have reported in many previous studies using this experimental system. This was expected based on reports that most inflammatory stimuli as well as ethanol are stressors.

All cytokines and chemokines tested were increased by poly I:C treatment, and they could be categorized by groups according to the association between corticosterone levels and changes in cytokine concentration. In the first group, cytokine/chemokine production (Fig. 4B) was inversely related to corticosterone concentrations (Fig. 4A). This association was particularly evident for IL-6. The production of IL-6 was enhanced substantially in the ADX groups. This also occurred in ethanol treated groups, but comparison of ADX + poly I:C group and the ADX + poly I:C + ethanol group indicates that inhibition of IL-6 production by ethanol is proportionally similar to inhibition of IL-6 in the absence of elevated corticosterone or catecholamine concentrations (in ADX mice). The results for all of the cytokines and chemokines in this first group and in our previous study demonstrate that the stress response to inflammatory stimulus markedly regulates the production of many cytokines, perhaps to prevent adverse effects of excessive cytokine concentrations. For most of these cytokines, it has been previously reported that glucocorticoids can inhibit their production. The second group, consisting of IL-10, IL-12 (p70), IFN- γ , and MIP-1 α , did not demonstrate any apparent correlation between corticosterone and cytokine levels (Fig. 4C). Acute ethanol treatment inhibited pro-inflammatory cytokine and chemokine production regardless of corticosterone concentration, even in ADX groups. Groups implanted with nadolol pellets did not demonstrate significant differences from their matching treatment groups (nadolol *vs.* sham, ADX + Nad + poly I:C *vs.* ADX + Placebo + poly I:C). This suggests that catecholamines acting through β 2-adrenergic receptors are not involved in regulating cytokine production in response to poly I:C. In addition, ethanol had the same effect on cytokine production with or without nadolol treatment for all cytokines and chemokines, indicating that catecholamines acting through β 2-adrenergic receptors are not involved in modulation of cytokine and chemokine production by ethanol. In addition, ethanol in ADX mice with corticosterone replacement yielded the same or greater suppression of cytokines/chemokines than observed in Sham ADX mice. If systemic epinephrine played a major role in this suppression, corticosterone alone should not have been sufficient to overcome the lack of both corticosterone and systemic epinephrine response in ADX mice. This is consistent with the results from mice treated with nadolol pellets in suggesting that catecholamines are not a major mediator of cytokine/chemokines production in this experimental system.

Results for IL-10 demonstrated no significant effects of ethanol and an influence of corticosterone only in the ADX group with a corticosterone pellet. This differed somewhat

from results in our previous studies, in which ethanol increased IL-10 production in poly I:C-treated mice. However, those earlier studies were performed using a poly I:C preparation that was subsequently found to be contaminated with a significant amount of endotoxin (Pruett *et al.*, 2004). In a recent comparison study in which both the contaminated preparation and an essentially endotoxin-free poly I:C preparation was used, the pattern of change of cytokine production caused by ethanol was similar. In contrast for IL-10, ethanol induced a significant increase when administered with the endotoxin-contaminated poly I:C, but not with the endotoxin-free poly I:C used in our current study (data not shown). Results for IL-1 β , IL-12, KC, GM-CSF, IL-6, and TNF- α indicate that loss of the corticosterone and/or systemic epinephrine response in ADX mice caused an increase in poly I:C-induced production of these molecules. Thus, stress mediators are involved in normal regulation of responses of these cytokines and serve to limit excessive production of these cytokines and chemokines.

Discussion

Acute ethanol exposure has been shown to be immunosuppressive, due at least in part to its ability to create a neuroendocrine stress response. This response includes the activation of the hypothalamic-pituitary-adrenal axis, which in mice, primarily culminates in elevated corticosterone concentrations (Carson and Pruett, 1996; Rivier *et al.*, 1984). The results reported here demonstrate that acute ethanol exposure augments the poly I:C-induced corticosterone response, whereas it attenuates the poly I:C-induced pro-inflammatory cytokine production. The increase in corticosterone levels following acute ethanol administration occurs more rapidly than the increase following poly I:C, which, interestingly, coincides with the optimal inhibition by ethanol of the early poly I:C-induced cytokines TNF- α and IL-6. These results at first seem to suggest that corticosterone is a major factor involved in the suppression of cytokines following acute ethanol exposure. However, this is not consistent with the results from ADX mice, since the suppression of cytokine/chemokine production due to ethanol occurs regardless of corticosterone levels, as demonstrated by comparing sham and ADX/placebo mice.

The adrenal gland is the site of both glucocorticoid and catecholamine synthesis. The adrenal cortex, derived embryologically from mesodermal mesenchyme, secretes steroid hormones, including glucocorticoids, whereas the adrenal medulla, derived from neural crest cells, secretes catecholamines, including epinephrine and norepinephrine (Ross *et al.*, 2003). In a stress response, the adrenal medulla releases epinephrine and norepinephrine in approximately a 4:1 ratio (Elenkov *et al.*, 2000). The primary source of norepinephrine in a stress response is from sympathetic nerve endings (Kvetnansky *et al.*, 1979). Therefore, adrenalectomized mice should have a strongly diminished corticosterone and epinephrine response, with only a slightly diminished norepinephrine response to stress. Virtually all lymphoid organs are innervated by sympathetic nerve endings, and all lymphoid cells except Th2 cells express β -adrenergic receptors (Sanders *et al.*, 1997). Expression of α -adrenergic receptors on lymphoid cells is an area of debate, but they are likely not expressed on lymphoid cells, except in certain pathologic conditions, or in certain populations, such as alveolar macrophages (Sanders *et al.*, 1997; Elenkov *et al.*, 2000). Therefore, a non-specific β -blocker such as nadolol should effectively block sympathetic stimulation of cells of the innate immune system.

Nadolol pellets placed in both ADX and non-ADX mice and corticosterone replacement pellets placed in ADX mice provide several lines of evidence that catecholamines do not play a major role in regulation of TLR3-induced cytokine responses or in the inhibition of cytokine production by ethanol. Mice in the ADX groups that received a corticosterone pellet had lower poly I:C-induced concentrations of cytokines than other groups, consistent with a corticosterone concentration greater than measured in all other groups (Fig. 4A). This indicates the importance of corticosterone in suppression of cytokine concentration in this system and

suggests that the lower, stress-induced level of corticosterone is sufficient to regulate most cytokines to some degree. The decrease in corticosterone in ADX mice is associated with an increase in poly I:C-induced cytokine concentrations. Two lines of evidence indicate that loss of epinephrine/norepinephrine in ADX mice does not contribute substantially to this effect. First, blocking the action of epinephrine/norepinephrine through β -adrenergic receptors using nadolol did not abrogate the effect of ADX. Second, in our previous study (Glover and Pruett, 2006), a glucocorticoid antagonist (RU 486) and a glucocorticoid synthesis inhibitor (aminoglutethimide) yielded similar effects on poly I:C-induced cytokine responses as ADX. However, these agents do not affect catecholamines, so the increased concentration of some cytokines caused by these agents or by ADX must be due to loss of corticosterone, not catecholamines. To our knowledge, this is the first report indicating that TLR3 mediated cytokine production is not substantially regulated by catecholamines acting via β -adrenergic receptors. Cytokine induction mediated by TLR4 has been reported to be regulated by catecholamines (Brown *et al.*, 1991; Ignatowski *et al.*, 1996; Hasko *et al.*, 1998b; Elenkov *et al.*, 1995; Meltzer *et al.*, 2004). A difference between TLR3 and TLR4 in this regard is not particularly surprising, because TLR3 lacks a major branch of the signal transduction pathway activated by TLR4, and this part of the pathway could be the target that allows regulation by catecholamines.

Results from ADX mice indicate that modulation of IL-10 production does not appear to be mediated by either arm of the neuroendocrine stress response nor by ethanol. It is not surprising that IL-10 does not follow the same pattern of response as the other cytokines and chemokines tested (i.e., IL-1 β , IL-6, IFN- α , GM-CSF, TNF- α , MIP-1 β , and KC) because these are generally considered pro-inflammatory under most conditions, whereas IL-10 is not. Interestingly, IL-12 (p70), IFN- γ , and MIP-1 α , however, are considered pro-inflammatory, and yet do not follow the same pattern of inhibition at elevated corticosterone levels as most others tested (note the ADX group with 5 mg corticosterone pellet in Fig. 4C). The molecular basis for this pattern of effects is not known. In fact, mechanisms of inhibition of relevant signaling pathways (e.g., NF- κ B) identified by investigators using pharmacological dosages of synthetic glucocorticoids were not identified in mouse T lymphocytes exposed to stress-inducible concentrations of corticosterone (Pruett *et al.*, 2003). Additional research is needed to characterize the mechanisms that are important in regulation of cytokine production by stress-inducible concentrations of glucocorticoids.

Both the HPA axis and the SNS play a role in immunoregulation associated with stressors. Since the two systems are so often induced together *in vivo*, use of *in vitro* and even *ex vivo* studies cannot adequately model the complexities of interactions *in vivo*. In addition, it has been demonstrated that not only viral and bacterial components, but also several cytokines, when injected systemically can increase either corticosterone, norepinephrine, or both differentially (summarized in Dunn, 2006). All of these factors make it difficult to separate the effects due to HPA versus SNS activation on immunosuppression in a whole animal. Adrenalectomy, corticosterone replacement, and a peripheral β -blocker were used in this study to examine the role of glucocorticoids and catecholamines in the ethanol-induced immunosuppression in mice stimulated with poly I:C. Although the HPA axis did appear to mediate suppression of proinflammatory cytokines in mice treated with poly I:C alone, it was not the primary mediator of the suppression of cytokine production caused by acute ethanol administration. Future studies in our lab will focus on the activation of transcription factors involved in TLR3 signaling and the effects of the stress response of acute ethanol administration on their activation.

The most commonly used synthetic agonist for TLR3 is polyinosinic polycytidylic acid (poly I:C), and this analog of double stranded RNA activates both murine and human cells. However, there is some evidence of differences in the regulation of TLR3 expression in these species

(Heinz *et al.*, 2003) and in the cell types that can be effectively activated to produce cytokines (Lundberg *et al.*, 2007). However, protection from some viral infections (e.g., Herpes simplex) is clearly mediated by TLR3 in humans as well as mice (Ashkar *et al.*, 2004; Bustamante *et al.*, 2008), and type I interferons are important products of TLR3 activation in both species (Svensson *et al.*, 2007; Matsumoto and Seya, 2008), suggesting that similar signaling pathways are induced and similar regulatory pathways can reasonably be expected. Although there are caveats, it would seem that the basic findings in this mouse model will probably be applicable in humans.

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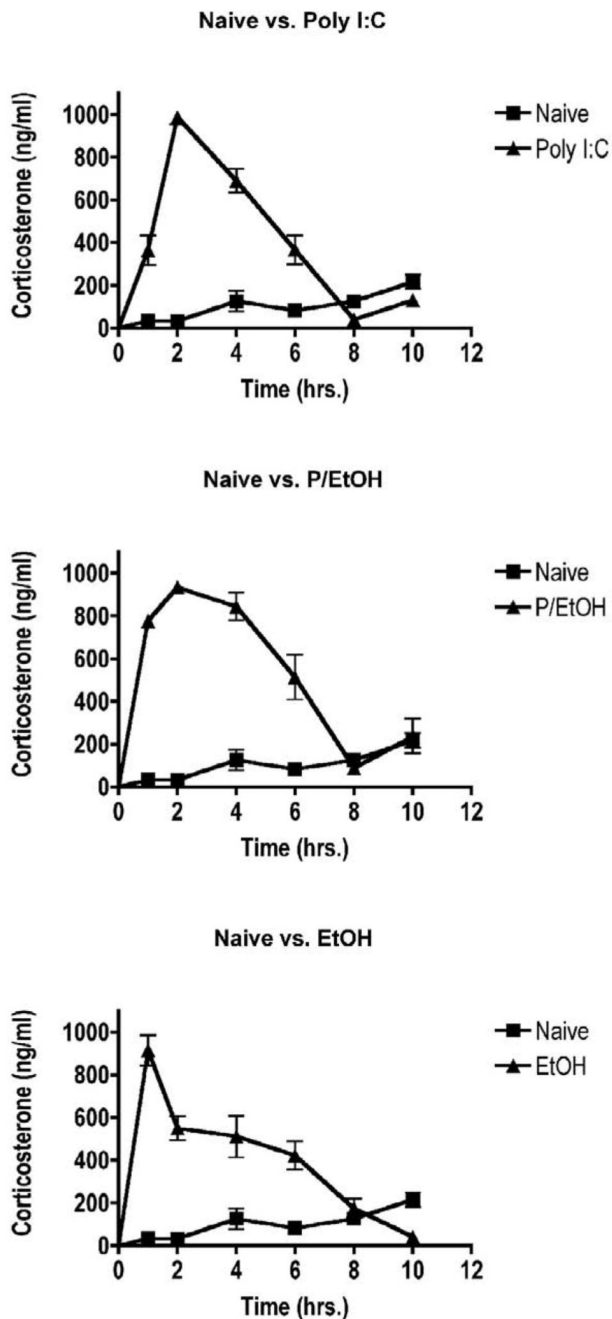


Figure 1. Corticosterone time course for calculation of the area under the concentration vs. time curve

Mice were treated with ethanol (6 g/kg by gavage) or vehicle (water) 15 minutes prior to poly I:C (100 μ g/mouse, intravenously). Naive groups received no treatment. Blood was collected from different groups (n = 5) at 1, 2, 4, 6, 8, and 10 hours after poly I:C dosing. Corticosterone levels were measured in serum by radioimmunoassay. Values shown are means \pm SEM.

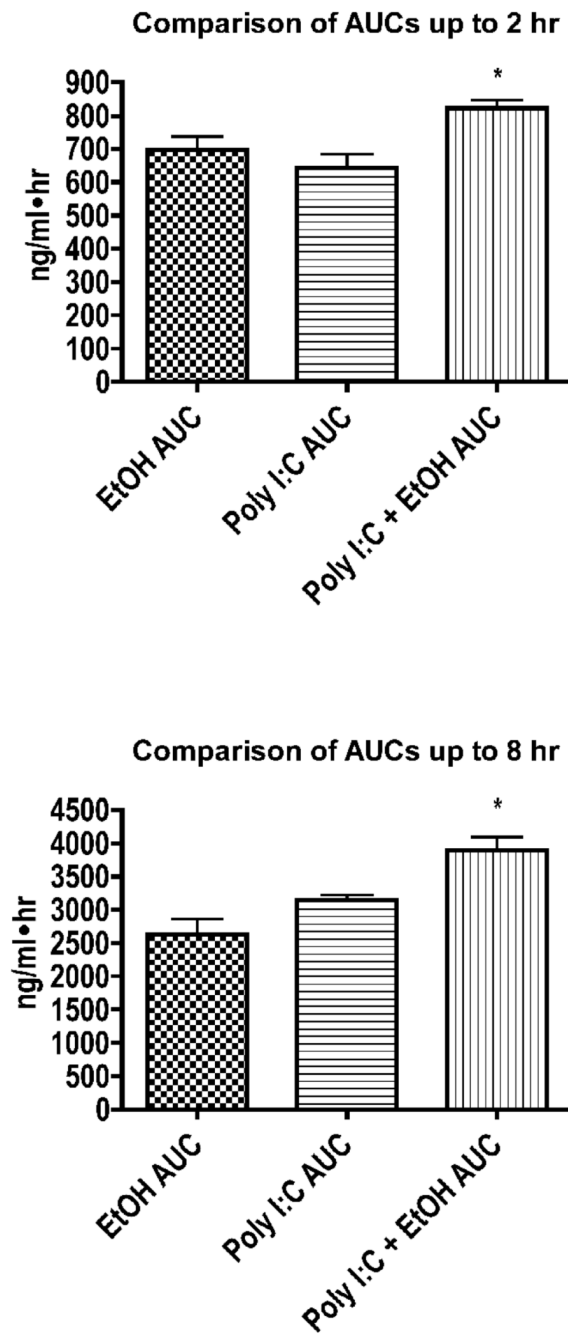


Figure 2. Comparison of areas under the corticosterone concentration vs. time curve (AUCs) Corticosterone concentrations from Figure 1 were used to calculate corticosterone AUCs (as described in Materials and Methods) *A*, up to 2 hours and *B*, up to 8 hours and these AUCs were compared using analysis of variance followed by Neuman-Keul's post hoc test. Values shown are mean AUCs \pm SEM ($n = 5$). The * indicates significant differences ($p < 0.05$).

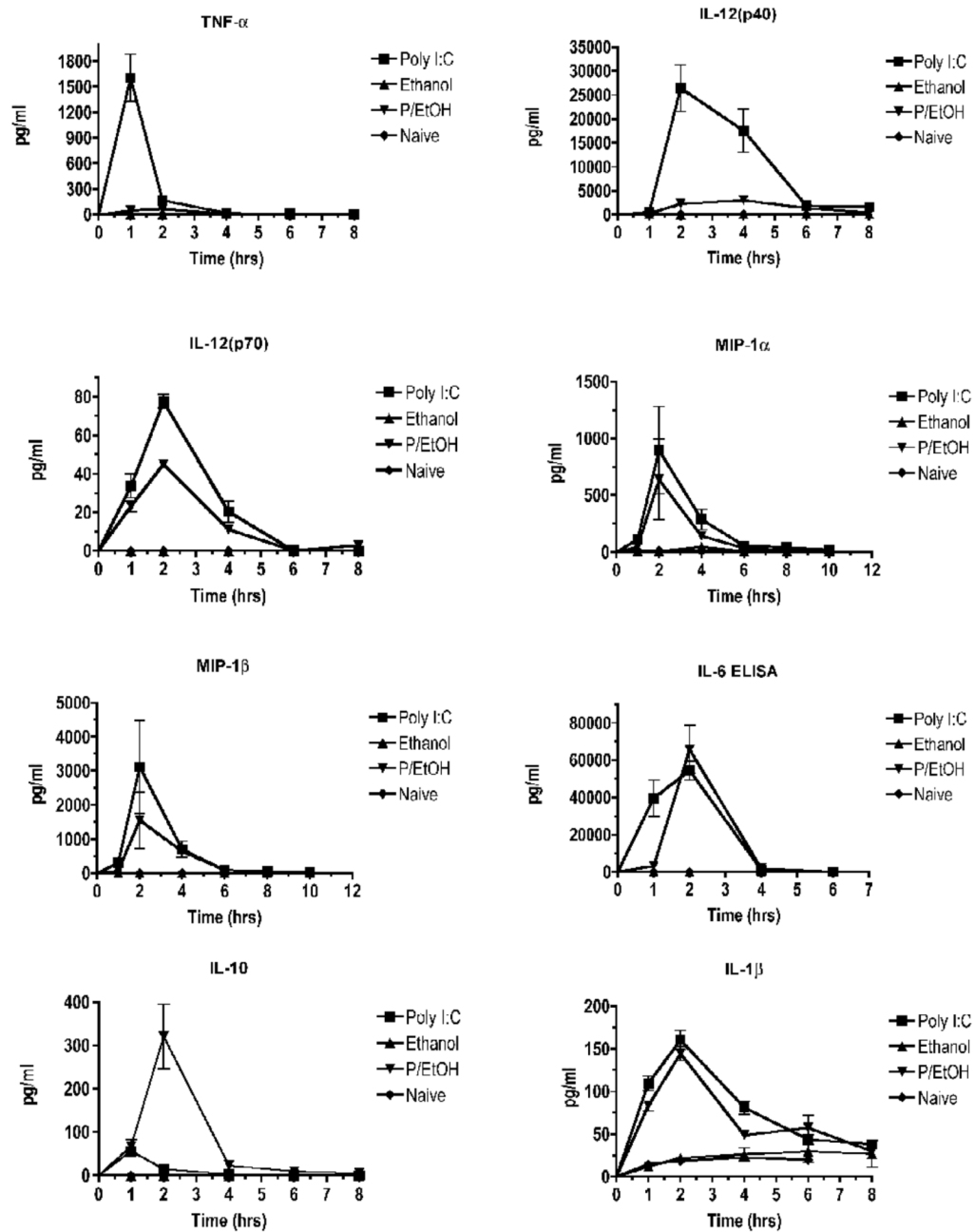
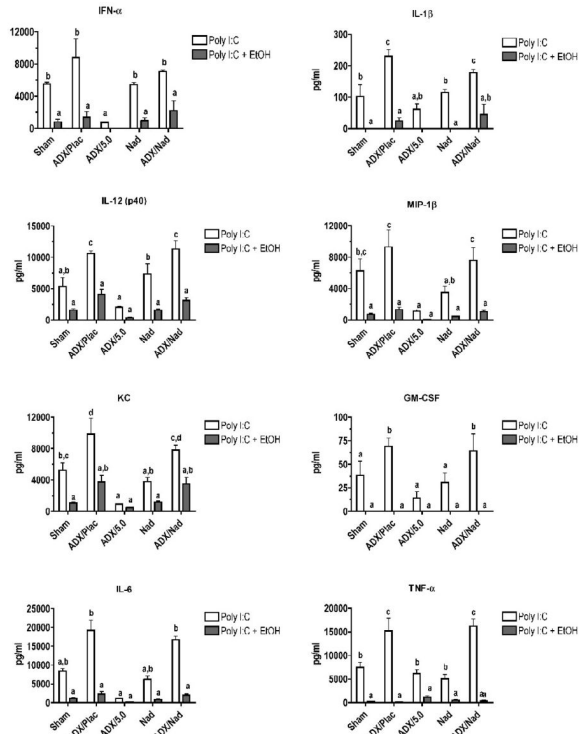
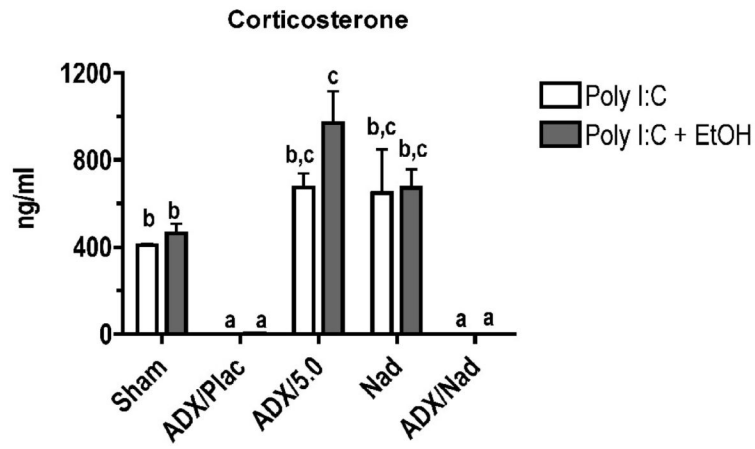


Figure 3. Cytokine time courses

Serum collected from mice used in the experiment shown in Figure 1 was used to measure cytokine concentrations for different treatment groups at a range of times after stimulation with poly I:C. Cytokine concentrations (except for IL-6 and IFN- α) were measured using Bioplex multiplex assays. IL-6 and IFN- α were measured by ELISA. Values shown are means \pm SEM ($n = 5$), and values that do not share any lowercase letters above the bar are significantly different ($p < 0.05$, by ANOVA and Newman-Keul's post hoc test).



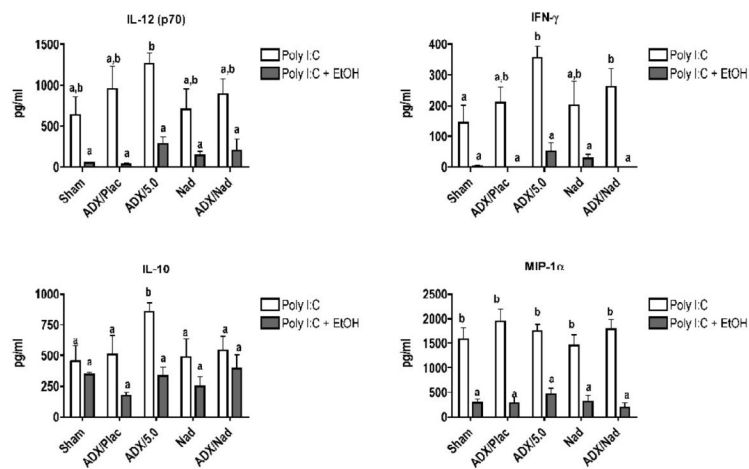


Figure 4. Effects of ADX on corticosterone and cytokine concentrations

Placebo, corticosterone (5.0 mg) and/or nadolol (0.5 mg) pellets were placed subcutaneously in ADX, sham, or untreated mice. One day later, mice were treated with ethanol (6 g/kg by gavage) or appropriate vehicle (water or 0.9% NaCl) 15 minutes prior to poly I:C (100 μg/mouse, intravenously) or vehicle (PBS). Blood was collected 2 hours after poly I:C administration. *A*) Serum corticosterone levels were measured by enzyme immunoassay. *B and C*) Cytokine levels (except for IL-6 and IFN-α) were measured using Bioplex multiplex assays. IL-6 and IFN-α were measured by ELISA. Values shown are means ± SEM. Results were analyzed by ANOVA followed by the Newman-Keuls post hoc test. The lowercase letters above the bars indicate the results of that analysis; groups that do not share any letter in common are significantly different ($p < 0.05$).