Ethanol's Effect on Rat Pituitary Adrenal Axis is Prevented by Purine Metabolic Pathway Inhibitors (44353)

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Abstract. **This study investigated the *in vitro* dose effects of ethanol (EtOH), adenosine (ADO), and urate (URA) on the basal and CRF stimulated ACTH production of pituitary tissue culture (PTC). Furthermore, the effects of low (LE = 0.5 g/kg bodyweight) and high (HE = 2.5 g/kg bodyweight) concentrations of EtOH were tested in rats on plasma ACTH and corticosterone concentration (PCC), with or without the following ADO metabolic pathway inhibitors: 6-mercaptopurine (6MP) (salvage pathway) and purpurogallin (PPG) (xanthine dehydrogenase).**

EtOH at 0–50 m*M* does not increase the *in vitro* basal or CRF (10^{-7} *M*) stimulated ACTH secretion in PTC; in fact doses up to 20 m*M* tended to be inhibitory. ADO significantly increased only basal ACTH secretion whereas URA increased both basal and CRF-stimulated ACTH secretion.

Pretreatment with PPG or 6MP + PPG significantly increased both *in vivo* ACTH and PCC over control values in rats. HE versus LE significantly increased ACTH and PCC in the control (H_2O) and 6MP pretreated groups whereas in the PPG pretreated animals, only ACTH was increased significantly by HE. However, combined pretreatment with 6MP + PPG prevented the effect of HE on ACTH and PCC. The current experiment suggests that purine metabolism is involved in ethanol's effect on the hypothalamic-pituitary-adrenal axis. [P.S.E.B.M. 1999, Vol 220]

High doses of ethanol (EtOH) *in vivo* have been shown to increase the production of adrenocorticotrophic hormone (ACTH) and plasma corticosterone (PCC) concentrations, whereas lower doses and chronic consumption have more varied effects (1–3).

In vitro, the acute effect of ethanol has been reported to increase (4), or leave unaltered spontaneous and CRF-stimulated ACTH release from pituitary cells, whereas pretreatment with EtOH for 24 hr decreased both the spontaneous and stimulated ACTH secretion (5). These varied effects of ethanol on ACTH secretion may be due to the length of exposure, the dose related metabolic differences, and the animal species tested.

Ethanol may influence the pituitary-adrenal axis either as an intact molecule, or its metabolites. Depending on the ethanol dose and the type of tissue, metabolites (i.e., acetaldehyde) may arise *via* three enzyme systems: alcohol dehydrogenase (ADH), catalase, and the microsomal ethanol oxidizing system (MEOS).

The metabolism of EtOH at low concentration (less than 30 m*M*) has been shown to be 70%–80% ADH dependent (6). Alcohol dehydrogenase is located predominantly in the liver; however, it has been detected in the cytoplasm of many other cell types, including the adrenals, the hypothalamus, and the infundibular stalk of the pituitary (7).

At higher ethanol concentration (>30 m*M*) and also in fasted rats, the contribution of catalase accounts for 50% of ethanol catabolism (8). Catalase and MEOS are found in the liver but have also been found in many other tissue types (9–11).

Acetaldehyde is further metabolized to acetate by NAD-dependent aldehyde dehydrogenase mainly in the mitochondria. The acetate molecule is activated by CoA and

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ATP, and acetyl-CoA and AMP are formed. Because of the high NADH/NAD ratio in the ADH containing cells, AMP is further catabolized to adenosine and its catabolic end product uric acid in humans and to uric acid/allantoin in rats.

Adenosine (ADO) and its metabolites have been shown to play an important role in the regulation of many physiological processes (12–14), one of which is ACTH-corticosterone signaling (15, 16). ADO occupies a central position in purine metabolism and has two primary metabolic pathways: a) reincorporation into the adenine nucleotide pool by phosphorylation or b) deamination to inosine and its subsequent metabolites (17). ADO (15, 16), uric acid (URA), and other purine metabolites (18, 19) have been shown to increase plasma ACTH and corticosterone concentration (PCC) significantly in rats. The interaction between EtOH, ADO, and its catabolic metabolites and the hypothalamic-pituitary-adrenal axis (HPAA) remains unclear. Therefore, we hypothesize that the stimulatory effect of EtOH on the HPAA is not only the direct effect of EtOH, but may be due to ethanol-induced increases of cellular ADO production (20) and/or inhibition of cellular ADO uptake (21); both mechanisms may result in increased extracellular ADO and/or URA concentrations and thereby stimulate ACTH production.

To elucidate the mechanism/s that may be involved in ethanol's effect on purine metabolic pathways as it relates to the HPAA, this study investigated: a) the *in vitro* effects of EtOH, ADO, and URA on the basal and CRF-stimulated ACTH production of pituitary primary cell cultures and b) the *in vivo* effects of low (LE = 0.5 g/kg bwt.) and high (HE = 2.5 g/kg bwt.) doses of ethanol on plasma ACTH and PCC, in rats, with or without combinations of 6-mercaptopurine (6MP) (salvage pathway) and purpurogallin (PPG) (xanthine dehydrogenase), both purine metabolic pathway inhibitors.

Materials and Methods

***In Vitro* Experiments.** Twenty, female, 160–200-g SPF rats (LATI, Gödöllõ, Hungary) were used for preparing the pituitary tissue cultures according to the method of Rappay *et al.* (22). The animals were anesthetized (Sodium Pentobarbital, 40 mg/kg), and the pituitaries were excised and placed into M199 tissue culture medium (Sigma Chemical Co., St. Louis, MO). The adenohypophysis was dissected, cut into pieces of 1–2 mm, incubated at room temperature in 25% trypsin (Difco) containing Tyrode's solution (Sigma) under constant stirring for 20 min, and the supernatant containing dispersed cells was removed and stored at 4°C. The supernatant fraction was pooled, centrifuged at 1000 *g* for 10 min, and then decanted; the residue was resuspended in 8:2 mixture of M199 and fetal calf serum (Sigma), supplemented with Penicillin (400 IU/ml). The pooled cell suspensions used for the experiments contained 6–8 $\times$ 10^5 cells/ml. From this suspension during constant stirring, exactly 1-ml portions were allocated per well (24 wells/plate, Tissue Culture Plates—Greiner Labortechnik, Gloucestershire, UK), assuring that equal numbers of cells were delivered per well. The deviation of cell numbers between wells was determined to be less than 7%. The viability of the cells was determined by light microscopic observation and showed no morphologic changes prior to or after incubation of the cells with the various substances tested. The plates were incubated at 37°C under 5% CO_2/air. The culture medium was changed 24 hr after the explanation of the cells, and thereafter every other day. A confluent monolayer was established by the 6th day of incubation and used for the experiment. At Time 0, the calf serum containing tissue culture medium was replaced by one of our treatment solutions and after 2 hr, the cell free supernatants were collected from each well, for ACTH determination. The samples were stored at −20°C.

The dose effect of EtOH at 5, 10, 20, 30, and 50 m*M* concentration on basal and CRF (10^{-7} mole final concentration)-stimulated ACTH secretion was studied as well as the effects of ADO and URA (sodium urate) at 10, 20, 40, 80, 160 and 200 μM concentrations. These doses were selected based on our previous work (18, 19) and other reported studies (16, 4), and the *in vitro* doses were comparable to doses administered *in vivo* (18). EtOH, ADO, and URA were dissolved in serum-free M199 tissue culture media; the pH of the test solutions was identical to the M199 control media. For each treatment, the average of 4 culture wells was reported.

***In Vivo* Experiments.** Male 160–200-g Wistar SPF rats (LATI, Gödöllõ, Hungary) were housed individually in wire-bottom cages, and the room temperature was maintained at 23°C. The animals were fed rat chow (NRC formulation, LATI, Gödöllõ, Hungary) and provided drinking water *ad libitum.* Animals were randomly advocated to the following twelve experimental groups as depicted in Table I (6 rats/group).

Pretreatments. 6-mercaptopurine (a hypoxanthine salvage pathway inhibitor) (8.5 mg), purpurogallin (xanthine dehydrogenase inhibitor) (50 mg), and the combination of these were suspended in 100 ml distilled water and of this solution 1 ml/100 g bwt was administered per os 2 hr prior to treatment. These concentrations have been shown to be effective inhibitors of these pathways (23–27). The control rats were pretreated with distilled water (1 ml/100 g bwt.).

Treatments. Five or 25 g ethanol was added to 0.9 g NaCl and adjusted with distilled water to 100 ml. Rats were given 1 ml/100 g bwt of these solutions intraperitoneally 2 hr after the administration of pretreatments. The same quantity of saline was given to the control animals.

One hour after intraperitoneal injection of the treatment solutions, animals were sacrificed. This time interval was selected based on our preliminary studies, which showed maximal ACTH and PCC responses to occur 60 min post-challenge. Sacrifice by decapitation occurred in 2-min staggered intervals between the various treatment groups to

Table I. Experimental Design

Groups	Pretreatment (po, 2 hr before treatments)	Treatments (ip)
Control	H_2O (1 ml/100 g bwt)	SAL (1 ml/100 g bwt)
Low ethanol (LE)	H_2O (1 ml/100 g bwt)	Ethanol (0.5 g/kg bwt)
High ethanol (HE)	H_2O (1 ml/100 g bwt)	Ethanol (2.5 g/kg bwt)
6-mercaptopurine (6MP)	6MP (8.5 mg/kg bwt)	SAL (1 ml/100 g bwt)
Purpurogallin (PPG)	PPG (50 mg/kg bwt)	SAL (1 ml/100 g bwt)
6MP-PPG	6MP + PPG (8.5 and 50 mg/kg bwt respectively)	SAL (1 ml/100 g bwt)
6MP-LE	6MP	Ethanol (0.5 g/kg bwt)
PPG-LE	PPG	Ethanol (0.5 g/kg bwt)
6MP-PPG-LE	6MP + PPG	Ethanol (0.5 g/kg bwt)
6MP-HE	6MP	Ethanol (2.5 g/kg bwt)
PPG-HE	PPG	Ethanol (2.5 g/kg bwt)
6MP-PPG-HE	6MP + PPG	Ethanol (2.5 g/kg bwt)

Note. 6MP = 6-mercaptopurine; PPG = purpurogallin

minimize any possible time effect due to circadian rhythm fluctuations between groups and to randomize the time of sacrifice among groups.

Biochemical Assays. Blood samples were collected in heparinized test tubes, and the plasma was separated by centrifugation at 3000 rpm for 10 min at 4°C and stored at −20°C until the determination of ACTH and PCC. Samples were not stored longer than 7 days prior to being assayed.

ACTH concentrations of the cell culture media and plasma were determined using a double antibody assay RIA kit (ICN Biomedicals, Inc., Costa Mesa, CA). The cross-reactivities of antisera with ACTH was 100%; with α-MSH, β-MSH, and β-endorphin, it was <0.1%, whereas with β-lipotropin it was <0.8%.

PCC was determined by RIA. The intra-assay variation of the corticosterone RIA was 6.4%. The final dilution of the corticosterone antibody was 1:40,000. Cross-reactivity of the antiserum with other steroids was <0.11%, except with progesterone (2.3%) and desoxycorticosterone (1.5%). The calibration curve ranged from 0.01 pmole to 20 pmole/tube.

Statistical Analysis. The *in vitro* data were analyzed by one-way ANOVA followed by LSD post-hoc test and the *in vivo* studies by two-way ANOVA followed by Tukey *post hoc* analysis to measure significant differences between treatment groups. Differences were considered to be significantly different at $P \leq 0.05$. The standard deviation of the difference between two means was calculated as follows:

$$\sqrt{(\text{Variation of group \#1/sample size}) + (\text{Variation of group \#2/sample size})}.$$

Results

In Vitro. Table II shows the dose effect of EtOH on basal and CRF-stimulated ACTH secretion of pituitary tissue culture cells (PTC). The acute exposure of PTC to different doses (5, 10, 20, 30, and 50 m*M*) of EtOH did not significantly alter baseline ACTH secretion; however, there was a trend toward decreased basal ACTH production at 20 m*M* EtOH.

Table II. Dose Effect of Ethanol on Basal and CRF-Stimulated ACTH Secretion of Pituitary Tissue Culture Cells

Ethanol (mmole/liter)	Basal ACTH Secretion (fmole/ml) Mean ± SD	CRF-Stimulated ACTH Secretion (fmole/ml) Mean ± SD
0	$46.34 \pm 24.99^{a,b}$	174.05 ± 35.00^{a}
5	$36.78 \pm 4.80^{a,b}$	104.03 ± 20.41^{b}
10	$34.23 \pm 4.47^{a,b}$	111.75 ± 25.56^{b}
20	21.95 ± 9.91^{b}	90.35 ± 18.54^{b}
30	47.38 ± 15.24^{a}	178.38 ± 21.19^{a}
50	$43.13 \pm 9.52^{a,b}$	167.33 ± 82.39^{a}

^{a,b} Mean values with different letters are significantly different at $P < 0.05$.

The CRF-induced ACTH secretion was significantly inhibited by low doses (5, 10, and 20 m*M*) of EtOH; there was no significant inhibition at doses equal to or higher than 30 m*M* EtOH.

ADO significantly increased basal ACTH secretion; a linear dose effect was observed to 20 μ*M* ADO concentration (Table III). ADO did not significantly alter CRF-stimulated ACTH secretion at the doses studied. URA significantly stimulated both the baseline and CRF-stimulated ACTH secretion of PTC (Table IV). The maximum effect of URA on basal ACTH secretion was noted at 10 μ*M* concentration whereas the CRF-stimulated maximal URA effect was noted at 40 μ*M* concentration. URA significantly decreased the basal ACTH secretion of PTC at 200 μ*M;* CRF stimulated ACTH secretion was not determined at this concentration.

In Vivo. Effect of pretreatments. As shown in Figure 1, animals pretreated with 6MP, compared to H_2O control, showed no significant differences in plasma ACTH and PCCs. However PPG and the combined pretreatment of

Table III. Dose Effect of Adenosine on Basal and CRF-Stimulated ACTH Secretion of Pituitary Tissue Culture Cells

Adenosine in the culture media (μmole/liter)	Basal ACTH secretion (fmole/ml) Mean ± SD	CRF-stimulated ACTH secretion (fmole/ml) Mean ± SD
0	62.1 ± 5.28[a]	243.5 ± 11.1
10	134.6 ± 10.6[b]	240.1 ± 26.4
20	182.9 ± 18.7[c]	268.4 ± 47.5
40	136.3 ± 10.8[b]	222.0 ± 32.9
80	157.5 ± 31.9[b,c]	268.9 ± 19.7
160	135.3 ± 23.1[b]	299.7 ± 40.4
200	58.3 ± 7.52[a]	—

[a,b,c] Mean values with different letters are significantly different at $P < 0.05$.

Table IV. Dose-Related Effect of Urate on Basal and CRF-Stimulated ACTH Secretion of Pituitary Tissue Culture Cells

Urate in the culture media (μmole/liter)	Basal ACTH secretion (fmole/ml) Mean ± SD	CRF-Stimulated ACTH secretion (fmole/ml) Mean ± SD
0	62.1 ± 5.28[a]	243.5 ± 11.1[a,b]
10	207.1 ± 43.3[c]	297.9 ± 40.2[a,b]
20	148.7 ± 29.4[b]	292.7 ± 53.4[a]
40	148.6 ± 34.6[b]	348.1 ± 18.4[d]
80	139.6 ± 50.0[b]	332.1 ± 13.6[c,d]
160	141.28 ± 28.8[b]	285.3 ± 31.9[b,c]
200	23.3 ± 5.16[d]	—

[a,b,c,d] Mean values with different letters are significantly different at $P < 0.05$.

6MP + PPG significantly increased plasma ACTH and PCC over the control.

Effect of ethanol. The effect of low (0.5 g = 10.86 mmole/kg bwt) and high doses (2.5 g = 54.3 mmole/kg bwt) of EtOH on plasma ACTH and PCC are shown in Figures 2 and 3. Neither plasma ACTH nor PCC was influenced significantly by the low dose of EtOH. However, the high dose of EtOH increased both plasma EtOH and PCC significantly.

Effect of pretreatment and ethanol. The group pretreated with 6MP, PPG, and 6MP + PPG, when given LE, did not show any significant changes on plasma ACTH concentration and PCC compared to pretreated control (Figs. 2 and 3).

In animals pretreated with 6MP or PPG and HE, plasma ACTH concentration significantly increased compared to the pretreated control. However, in animals that were pretreated with co-administered 6MP + PPG, the effects of HE on plasma ACTH were prevented (Fig. 2).

Animals pretreated with 6MP showed significant increase in PCC after HE treatment. PCC was not further increased by HE in the animals pretreated with PPG or 6MP + PPG (Fig. 3).

Two-way ANOVA showed that there were significant pretreatment effects, treatment effects, and a significant interaction between pretreatment and treatment for both ACTH and PCC.

Discussion

In Vitro. The *in vitro* data clearly demonstrated that EtOH even at high doses (30–50 m*M*) does not increase basal or CRF-stimulated ACTH secretion. In fact doses up to 20 m*M* tended to inhibit both basal and CRF-stimulated secretion of pituitary cells. This observation is identical to the results reported by Rivier *et al.* (5), but it is in contrast to the results obtained from a superfused pituitary fragment system by Redei *et al.* (4). The most likely explanation for the difference is the time interval (approximately 8 min) used in the studies reported by Redei *et al.* (4) compared to our 2-hr incubation period. However, if the low dose of EtOH were in fact stimulatory, we would also expect the concentration of ACTH to increase in our pituitary cell cultures; however, this was not the case. Perhaps the pulsatile nature of ACTH secretion while initially being increased, may also, with time, result in decreased secretion and therefore the effect, during the 2-hr period, may result in no net change or a negative balance. An alternative explanation could be that the pituitary culture does not metabolize EtOH. If EtOH is not metabolized, it will not increase the ADO or URA indirect end products, which clearly stimulate ACTH secretion (Tables III and IV). The biphasic dose response effect of ADO (Table III) may be due to different receptor saturation kinetics at the high dose. A possible toxic effect of the ADO high dose, while unlikely, cannot be excluded. These questions cannot be answered based on the current studies. Others have also shown a dose response of ACTH to ADO at low concentrations (16); however, the effect of ADO at 200 μ*M* has not been reported.

In Vivo. The results of our *in vivo* studies clearly showed that high versus low doses of EtOH increased plasma ACTH and PCC, and these findings are in agreement with other reported studies (1–3). Our *in vitro* and *in vivo* studies, as well as studies reported by others (5, 28), suggest that the stimulatory effects of EtOH on the hypothalamic-pituitary-adrenal axis are most likely mediated at the hypothalamus and not at the pituitary level. However, the purine metabolites, ADO and URA, which result indirectly from EtOH catabolism, clearly stimulate ACTH secretion at the pituitary level (18, 19). The hypothalamus has been shown to contain both alcohol dehydrogenase (7) and catalase (29). We hypothesize that the metabolism of LE *via* primarily alcohol dehydrogenase, which is rate limiting, does not yield effective concentrations of ADO or URA to stimulate A_2 receptors. However when EtOH is present at high concentrations, it can be catabolized more rapidly by the catalase-H_2O_2 system, which has been found in the brain (30), and hypothalamus (29), and high ethanol concentration inhibits the cellular re-uptake of ADO (21). The alter-

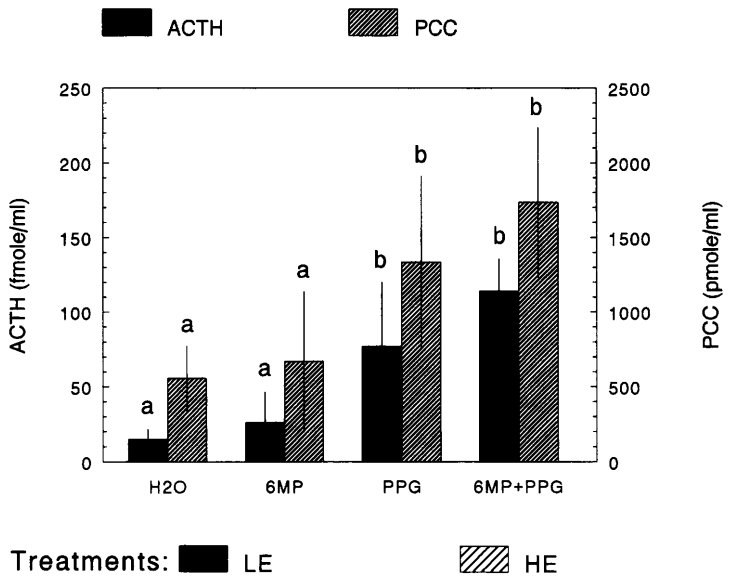

Figure 1. The effects of pretreatment on ACTH and plasma corticosterone concentration (PCC) compared to saline control (n = 6/treatment group). 6MP = 6-mercaptopurine. PPG = purpurogallin. * = values are significantly different ($P < 0.05$) from H_2O-saline control values. Values are mean ± SD.

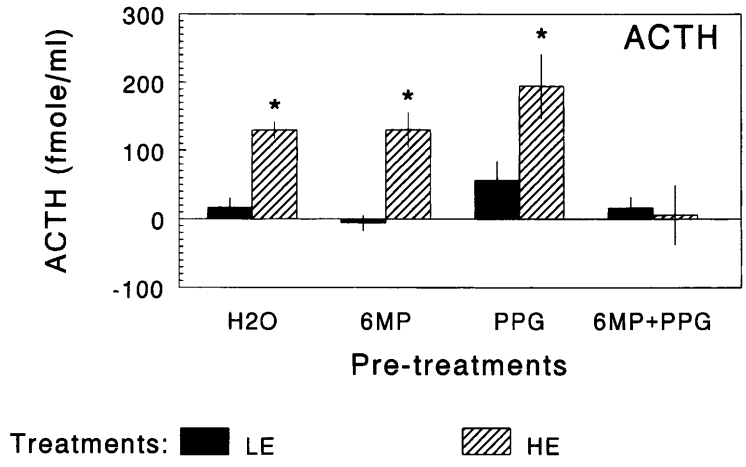

Figure 2. Effect of EtOH on plasma ACTH concentration in rats (n = 6/treatment group). The figure shows the ethanol + pretreatment effect minus the pretreatment effect. 6MP = 6-mercaptopurine. PPG = purpurogallin. * = values are significantly different ($P < 0.05$) from the pretreated values. Values are differences of the means ± SD.

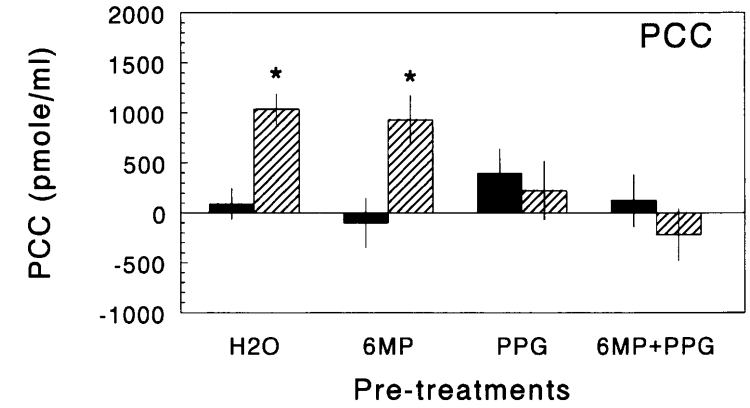

Figure 3. Effect of EtOH on plasma corticosterone concentration in rats (n = 6). The figure shows the ethanol + pretreatment effect minus the pretreatment effect. 6MP = 6-mercaptopurine. PPG = purpurogallin. PCC = plasma corticosterone concentration. * = values are significantly different ($P < 0.05$) from the pretreated values. Values are differences of the means ± SD.

ation of these regulatory pathways may yield effective concentrations of ADO and URA for altering the HPAA.

The current results, as well as previous *in vivo* data, clearly show that the purine metabolites, ADO (16, 18, 31) and URA (18), stimulate the hypothalamic-pituitary-adrenal axis. Although in these studies we did not measure intra- or extracellular ADO or URA concentrations, it has been demonstrated by others, in human and other animal models, (20, 32) that the metabolism of high concentrations of EtOH results in increased ADO and/or URA production. Further studies are needed to clearly establish that 6MP + PPG increase extracellular concentrations of ADO and/or URA.

Under normal physiological conditions the extracellular ADO concentration most likely does not reach levels

capable of eliciting an A_2 receptor because a) ADO produced by the peripheral tissues is rapidly catabolized to URA by the endothelial cells (33) prior to reaching the central nervous system and b) its half-life in the blood is short, 0.1–1.6 sec (34). EtOH metabolism also results in a high NADH/NAD ratio (35) and increased acetate concentration in the liver and also in the systemic circulation (36). Both EtOH and acetate increase purine nucleotide degradation by enhancing the turnover of the adenine nucleotide pool (20). This may contribute to the increased ADO and URA concentration either locally in the hypothalamus or in the systemic circulation. Sumi and Umeda (37) have shown that ventromedial stimulation of the hypothalamus increases URA production.

EtOH at high doses not only increases the catabolism of purine nucleotides, but also inhibits the re-uptake of ADO by the cells (21); thus, it creates a metabolic situation in which the extracellular concentration of ADO may reach the threshold level needed for A_2 receptor stimulation.

It is clear from the *in vivo* studies that inhibitors of purine metabolism markedly influence plasma ACTH and PCC. Neither of the pretreatments alone was inhibitory to ACTH or corticosterone secretion; in fact, the PPG and 6MP + PPG pretreatment significantly increased the plasma levels of these hormones (Fig. 1).

The inhibition of either the salvage or the hypoxanthine dehydrogenase pathway independently did not prevent the EtOH-induced increase of plasma ACTH. However, 6MP + PPG together prevented the specific EtOH effect (Figs. 2 and 3), suggesting that both pathways play an important role in controlling the hypothalamic-pituitary-adrenal axis.

The independent effect of the inhibitors might be explained by one of the following: a) 6MP by blocking only the hypoxanthine salvage pathway may significantly increase the plasma URA concentration to a level that can stimulate the hypothalamic-pituitary-adrenal axis by an unknown mechanism or b) PPG by inhibiting xanthine dehydrogenase may increase the hypoxanthine salvage to IMP and AMP. Since the AMP cannot be rephosphorylated because of the high NADH/NAD ratio and low P_i (32) concentration in the ethanol metabolizing cells, it will be catabolized to ADO or inosine. Thus, leaking of ADO from the ethanol-treated cells may be enhanced and its re-uptake inhibited, again contributing to a metabolic situation in which the extracellular concentration of ADO may reach the threshold needed for A_2 receptor stimulation. Additionally, inhibiting both pathways most likely results in hypoxanthine accumulation which, as we have shown previously, does not stimulate ACTH (18) secretion.

An interesting observation is that HE treatment did not increase the corticosterone concentration of the PPG pretreated animals over the pretreated control, whereas the plasma ACTH significantly increased. A possible explanation for this phenomenon may be that PPG inhibits purine degradation, which results from EtOH catabolism in the adrenal and therefore increases local ADO concentrations. ADO has been shown to inhibit basal and ACTH-stimulated corticosterone secretion (16).

The differences noted between the *in vitro* and *in vivo* effects of EtOH are most likely due to the incomplete metabolism of EtOH under *in vitro* conditions versus *in vivo*. The current experiment and past studies (15, 16, 18, 19) suggest that purine metabolism and the resultant metabolites (ADO and URA), which are increased during *in vivo* EtOH metabolism, are involved in the regulation of EtOH's effect on the hypothalamic-pituitary-adrenal axis.

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