ACETALDEHYDE, A METABOLITE OF ETHANOL, ACTIVATES THE HYPOTHALAMIC–PITUITARY–ADRENAL AXIS IN THE RAT

HIROSHI KINOSHITA, DAVID S. JESSOP, DAVID P. FINN, TONI L. COVENTRY, DAVID J. ROBERTS1, KIYOSHI AMENO2, IWAO JIRI2 and MICHAEL S. HARBUZ*

URC for Neuroendocrinology, University of Bristol, BRI, Marlborough Street, Bristol BS2 8HW, 1 School of Chemistry, University of Bristol, Cantocks Close, Bristol BS8 ITS, UK and 2Department of Forensic Medicine, Kagawa Medical University, 1750-1, Miki, Kita, Kagawa, 761-0793, Japan

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Abstract — Cyanamide is a potent inhibitor of aldehyde dehydrogenase (ALDH: EC 1.2.1.3) used in the treatment of alcoholics. In the presence of ethanol, cyanamide causes an accumulation of acetaldehyde, a highly toxic metabolite of ethanol, with unpleasant side effects. A similar accumulation is seen in some Oriental people with low ALDH activity. We have investigated the effects of ethanol and cyanamide administration on the activation of the hypothalamic–pituitary–adrenal (HPA) axis using in situ hybridization histochemistry and radioimmunoassay. Ethanol plus cyanamide resulted in a significant increase in corticotrophin-releasing factor and arginine vasopressin mRNA in the paraventricular nucleus, and pro-opiomelanocortin mRNA in the anterior pituitary. Plasma corticosterone concentrations were also significantly elevated following ethanol plus cyanamide administration. The blood concentration of acetaldehyde in the ethanol plus cyanamide group increased significantly. These results suggest that acetaldehyde, induced by blocking ethanol metabolism, is able to activate the HPA axis operating through a central mechanism.

INTRODUCTION

Ethanol has several actions on the central nervous system believed to be mediated by non-specific physicochemical effects on the membrane or by actions through specific receptors (Deitrich et al., 1989; Eckardt et al., 1998). Ethanol has a variety of effects on neuroendocrine function and there is a great deal of interest in investigating the effects of ethanol on the hypothalamic–pituitary–adrenal (HPA) axis. Ethanol administration activates the HPA axis (Ellis, 1966; Rivier et al., 1984; Rivier and Vale, 1988; Thiggarjan et al., 1989; Rivier, 1996; Rivier and Lee, 1996; Ogilvie et al., 1997), similar to that seen in response to stress (Harbuz and Lightman, 1997). It is generally accepted that, in response to acute stress, corticotrophin-releasing factor (CRF) and arginine vasopressin (AVP) are increased within parvocellular cells of the paraventricular nucleus (PVN). Approximately 50% of the CRF-containing cells of the PVN also contain AVP under normal conditions (Whitnall et al., 1985, 1987); the number of AVP-containing CRF cells is increased following stress (Whitnall, 1989). CRF and AVP are released from axon terminals in the external zone of the median eminence into the hypophyseal portal blood. CRF is currently the only releasing factor demonstrated to stimulate synthesis of pro-opiomelanocortin [POMC; the adrenocorticotropic hormone (ACTH) precursor] gene expression in the anterior pituitary (Levin et al., 1989). CRF and AVP act synergistically to release ACTH from the anterior pituitary, which stimulates the synthesis and release of corticosterone from the adrenal cortex (Gillies et al., 1982; Rivier and Vale, 1983; Harbuz and Lightman, 1997).

Ethanol is metabolized in the liver by three pathways: alcohol dehydrogenase (ADH), the microsomal ethanol-oxidizing system (MEOS) and catalase (Eckardt et al., 1998). Acetaldehyde, the first metabolite of ethanol, is produced in the liver following the first step of ethanol metabolism and is ten times more toxic than ethanol (Brien and Loomis, 1983). Most acetaldehyde is oxidized quickly to acetate by aldehyde dehydrogenase (ALDH), and its concentration in blood is very low (Eckardt et al., 1998). However, in some Oriental peoples with a genetically lower activity of ALDH, high blood concentrations of acetaldehyde are produced following ethanol ingestion (Mizoi et al., 1979; Harada et al., 1981; Enomoto et al., 1991) and this can result in stressful adverse effects such as nausea, headache, hypotension and palpitations. This rapid accumulation of acetaldehyde in blood following ethanol ingestion is believed to play a protective role against alcoholism (Harada et al., 1982). Acetaldehyde appears to mediate some of the behavioural and central neurotoxic effects of ethanol (Hunt, 1996), but the details of its properties are still obscure (Brien and Loomis, 1983).

Cyanamide is a potent inhibitor of ALDH (Deitrich et al., 1976), used for the treatment of alcoholism in some countries (Sellers et al., 1981; Valerdiz and Vazquez, 1989). When cyanamide is taken with ethanol, it produces severe noxious symptoms due to the accumulation of acetaldehyde (Sellers et al., 1981; Paechy and Naranjo, 1984). Cyanamide has a rapid onset of ALDH inhibition and long duration of action following i.v. administration (Deitrich et al., 1976; Loomis and Brien, 1983), and has been used as an animal model to mimic the lower capacity of acetaldehyde oxidation seen in some oriental people (Shinohara et al., 1993; Kinoshita et al., 1995, 1996). Acute administration of cyanamide alone can induce HPA axis activation in a dose-dependent manner (Kinoshita et al., 2000).

The present study was designed to determine the acute effects of ethanol and cyanamide interactions on HPA axis activation. We chose to use a dose of cyanamide insufficient to stimulate the HPA axis alone. The results indicate that these interactions stimulate the HPA axis through a central mechanism.

MATERIALS AND METHODS

Adult male Sprague–Dawley rats weighing 220–270 g were used. They were housed in a temperature- and

*Author to whom correspondence should be addressed.
humidity-controlled environment and maintained on a 12 h light:12 h darkness cycle. All rats had free access to food and water. All procedures were carried out according to Home Office (UK) guidelines.

Three days prior to the study, all animals were implanted with an intravenous (i.v.) cannula in the jugular vein to allow drug administration and blood sampling in freely moving animals. Following surgery, animals were housed in individual cages. Rats were handled daily to habituate to the experimental procedure and minimize the effects of handling. The lines were flushed daily with heparin saline (25 U/ml) to maintain patency. All experiments were started between 09.30 and 10.00 h. At the time of experiment, the i.v. cannula was connected to a 1 ml syringe filled with heparinized saline.

Four experimental groups were used: saline (as control), cyanamide alone, ethanol alone and ethanol plus cyanamide. Rats received an i.v. injection of cyanamide (Sigma Chemical Co., Dorset, Poole, UK) at a dose of 10 mg/kg or saline in a volume of 0.1 ml/100 g body wt 60 min before ethanol administration. Ethanol was administered by intraperitoneal (i.p.) injection at a dose of 1.0 g/kg adjusted by body wt to an 8% concentration of ethanol in saline solution to prevent peritoneal irritation (Rivier, 1996). Further control and cyanamide groups received i.p. injections of saline.

Blood samples (250 µl) were taken immediately prior to i.v. injection of cyanamide (time –60 min), just before ethanol administration (time 0), and at 30 min and 60 min following administration of ethanol. After each blood sampling, an equal volume of heparinized saline was infused. Animals were killed by decapitation 4 h following ethanol administration (5 h after cyanamide administration). The 4 h time-point has previously been shown to be a suitable one to demonstrate a significant increase in CRF mRNA after stress (Harbuz and Lightman, 1989). Brains and pituitaries were rapidly removed, frozen on dry-ice and stored at –80°C until sectioning. Sections (12 µm thick), containing the medial paraventricular region of PVN or pituitary, were cut and thaw-mounted on gelatin-coated slides and stored at –80°C before hybridization. Trunk blood was collected into ice-cold heparinized tubes. All blood samples were centrifuged and the plasma stored at –20°C prior to assay for corticosterone.

In situ hybridization histochemistry (ISHH)

ISHH was performed as described previously (Young et al., 1986a; Harbuz and Lightman, 1989; Harbuz et al., 1991). In brief, the sections were warmed at room temperature for 10 min, fixed with 4% formaldehyde for 5 min, washed twice in phosphate-buffered saline, and then incubated in 0.25% acetic anhydride in 0.1 M triethanolamine/0.9% NaCl for 10 min, and passed through 70% (1 min), 80% (1 min), 95% (2 min) and 100% (1 min) ethanol, 100% chloroform (5 min), and 100% (1 min) and 95% (1 min) ethanol before drying in air.

The probes used were 48-mer oligonucleotides complementary to part of the exonic mRNA sequences (Perkin-Elmer, Warrington, UK). The specificity of these probes has been determined previously (Young et al., 1986a,b; Levy and Lightman, 1988). The probes were labelled at the 3′ end with 35S-labelled deoxy-ATP (1000 µCi/mmole, NEN, Boston, MA, USA) using terminal deoxynucleotidyl transferase (Boehringer-Mannheim, Lewes, Sussex, UK) and column-purified by QIA quick nucleotide removal kit (Qiagen Ltd, West Sussex, UK). The specific activities of the probes were 4.95 × 1018, 3.14 × 1018 and 1.87 × 1018 d.p.m./mmol for CRF, POMC and AVP, respectively. Approximately 100 000 c.p.m. probes (per 45 µl) were applied to each slide. Hybridization was performed overnight at 37°C. All the sections for each hybridization were performed at the same time. The sections were washed in four 15 min rinses of 1 × SSC (1 × SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) at 55°C, followed by two 30 min washes in 1 × SSC at room temperature to remove non-specific binding before two short water rinses and then air-dried.

The sections, together with 14C-labelled standard (ARC146C; American Radiolabeled Chemicals Inc., St Louis, MO, USA) were exposed to Hyperfilm MP autoradiography film (Amersham International plc, Amersham, Bucks, UK). The autoradiographic image was measured as previously described (Harbuz et al., 1994; Kinoshita et al., 2000) using a computer-assisted image analysis system (Image 1.22, developed by W. Rasband, NIH, Bethesda, MD, USA) run on Apple Macintosh. The results are presented as the mean percentage change from control, assigned an arbitrary value of 100, as the mean ± SEM for each group.

Radioimmunoassay

Total plasma corticosterone was measured (3 µl plasma diluted in 100 µl buffer) using antisera kindly supplied by Dr G. Makara (Institute of Experimental Medicine, Budapest, Hungary). The tracer was [125I]corticosterone (ICN Biomedicals, Irvine, CA, USA) with a specific activity of 2–3 mCi/µg. The sensitivity of the assay was 10 ng/ml.

Gas chromatography

The concentrations of ethanol and acetaldehyde in plasma were measured simultaneously by a head-space gas chromatography method described previously (Okada and Mizoi, 1982).

Statistics

The data are expressed as mean ± SEM. All groups within each data set were compared by one-way ANOVA followed by Fisher PLSD test for multiple comparisons. A value of  P < 0.05 was considered significant.

RESULTS

The time-course effects of ethanol plus cyanamide on plasma corticosterone concentration are shown in Fig. 1. The i.p. administration of saline (control) at time 30 min caused a small increase in corticosterone, which could be ascribed to the stress caused by handling and injection, but no effect at any other time-points. The cyanamide-injected groups produced a slight, but not significant, increase in plasma corticosterone at 0 and 30 min. Ethanol produced a rapid increase in plasma corticosterone within 30 min [one-way ANOVA: F(3, 23) = 35.61,  P = 0.0001;  P < 0.01 vs control and cyanamide], with a return to basal level after 240 min. Ethanol plus cyanamide produced a dramatic increase in plasma corticosterone at 30 and 60 min [one-way ANOVA: F(3, 23) = 16.29,  P = 0.0001 at 60 min;  P < 0.001 vs control, cyanamide and ethanol at both 30 and 60 min], and it remained
The changes in CRF mRNA in the PVN 4 h after ethanol administration are shown in Fig. 2. These are expressed as a percentage of control group. We found a significant increase in CRF mRNA in the ethanol plus cyanamide group [one-way ANOVA: $F(3, 27) = 3.22, P < 0.01$ vs control, $P < 0.05$ vs cyanamide and ethanol, respectively]. The levels of AVP mRNA in the PVN are shown in Fig. 3. As with CRF, AVP mRNA was significantly increased only in the ethanol plus cyanamide group [one-way ANOVA: $F(3, 27) = 4.005, P = 0.0176$; $P < 0.01$ vs control, $P < 0.05$ vs cyanamide and ethanol, respectively].

The levels of POMC mRNA in the anterior pituitary were significantly increased only in the ethanol plus cyanamide group (Fig. 4) [one-way ANOVA: $F(3, 27) = 3.103, P = 0.0432$; $P < 0.05$ vs control and cyanamide].

Peak concentrations of ethanol and acetaldehyde are shown in Table 1. Ethanol concentrations were similar in both groups. In contrast, the acetaldehyde concentration dramatically increased in the ethanol plus cyanamide group [one-way ANOVA: $F(1, 13 = 18.812, P = 0.001; P < 0.001$ vs ethanol]. Ethanol and acetaldehyde concentrations in control and cyanamide-treated rats were below the detection limits.

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**Fig. 1.** Time course of plasma corticosterone concentrations (ng/ml) in rats following administration of saline (CONTROL), cyanamide (CY), ethanol (EtOH) and ethanol plus cyanamide (CY + EtOH).

Values are presented as means ± SEM for $n = 6–8$ rats/group. ${}^* P < 0.05$ compared with CONTROL and CY. **${}^** P < 0.01$ compared with CONTROL and CY, and §§ $P < 0.001$ compared with CONTROL, CY + EtOH.

**Fig. 2.** Corticotrophin-releasing factor (CRF) mRNA levels in the paraventricular nucleus of rats following administration of saline (CONTROL), cyanamide (CY), ethanol (EtOH) and ethanol plus cyanamide (CY + EtOH).

CRF mRNA levels are expressed as the percentage change from the control group. Values are presented as means ± SEM for $n = 7–8$ rats/group. ${}^* P < 0.05$ compared with CY and EtOH, and **${}^{**} P < 0.01$ compared with CONTROL.

**Fig. 3.** Arginine vasopressin (AVP) mRNA levels in paraventricular nucleus of rats following administration of saline (CONTROL), cyanamide (CY), ethanol (EtOH) and ethanol with cyanamide (CY + EtOH).

AVP mRNA levels are expressed as the percentage change from control group. Values are presented as means ± SEM for $n = 7–8$ rats/group. ${}^* P < 0.05$ compared with CY and EtOH, and **${}^{**} P < 0.01$ compared with CONTROL.

**Fig. 4.** Pro-opiomelanocortin (POMC) mRNA levels in the anterior lobe of the pituitary of rats following administration of saline (CONTROL), cyanamide (CY), ethanol (EtOH) and ethanol plus cyanamide (CY + EtOH).

POMC mRNA levels are expressed as the percentage change from the control group. Values are presented as means ± SEM for $n = 7–8$ rats/group. ${}^* P < 0.05$ compared with CONTROL and CY.
DISCUSSION

We have investigated the effects of acetaldehyde on the HPA axis using ethanol plus cyanamide. We present novel data demonstrating that ethanol plus cyanamide, a treatment which increases the concentration of acetaldehyde in blood, resulted in activation of the HPA axis at all levels of the axis. CRF and AVP mRNA in the PVN and POMC mRNA in the anterior pituitary increased significantly following ethanol plus cyanamide administration. The response observed in the ethanol plus cyanamide group was similar to a typical stress response through to the endpoint of HPA axis activation, i.e. an increase in corticosterone from the adrenal gland (Harbuz and Lightman, 1997). Plasma concentrations of corticosterone were increased significantly at 30 min in the ethanol group compared to control, and the corticosterone concentration of the ethanol plus cyanamide group was significantly increased after 30 min compared to control, cyanamide and the ethanol-alone group.

It has been established that with the moderately intoxicating dose of 3 g/kg of ethanol given i.p. or intragastrically, there is activation of the HPA axis with increased plasma concentration of ACTH (Rivier and Lee, 1996; Ogilvie et al., 1997). Activation of CRF and AVP mRNA in the PVN suggests a direct action of ethanol on the central nervous system. However, the dose of ethanol which we used in this experiment (1 g/kg) is an anxiolytic dose (Eckardt et al., 1998), with little effect on HPA axis activation (Ogilvie et al., 1997). A threshold blood alcohol concentration of 0.1–0.14% (w/v) has been reported as necessary for corticosteroid release in humans and mice (Jenkins and Connolly, 1968; Pruett et al., 1998). The difference of reported threshold concentrations may be due to the difference in the subjects. In addition, in these reports, the blood levels of acetaldehyde were not considered. This may be a pertinent point. The peak concentration of ethanol in our experiment was ~0.11%, the serum corticosterone concentration was moderately elevated in the ethanol group, and dramatically elevated in the ethanol plus cyanamide group at the 30 min time-point. CRF and AVP mRNA in the parvocellular PVN and POMC mRNA in the anterior pituitary were significantly increased only in the ethanol plus cyanamide group. The present results suggest that low doses of ethanol combined with cyanamide may exert direct effects on central nervous system functioning, due to the high concentration of acetaldehyde accumulated in blood. In other words, acetaldehyde has a strong potential to stimulate at all levels of the HPA axis. It has previously been reported in a perfusion study (Cobb et al., 1981) that ethanol and acetaldehyde can have a direct stimulatory action on the adrenal gland. In the present study we have noted a significant increase in plasma corticosterone within 30 min which may be as a result of a direct action at the level of the adrenal gland. However, the increase in CRF mRNA and AVP mRNA in the PVN suggests that these effects are centrally mediated and reflect the increase in CRF and AVP release into the hypophyseal portal blood stimulating ACTH release from the anterior pituitary, which then stimulates the release of corticosterone from the adrenal gland. The increase in POMC mRNA observed in the anterior pituitary reflects stimulation by CRF (Levin et al., 1989).

In this experiment, we used cyanamide as a potent ALDH inhibitor to produce an accumulation of acetaldehyde in blood. An alternative method to determine acetaldehyde effects on the HPA axis would be to investigate acetaldehyde directly. However, direct injection of acetaldehyde i.p. is problematic due to the intense irritability of acetaldehyde, even at low concentrations. The low doses of ethanol (1 g/kg, i.p.) and cyanamide (10 mg/kg, i.v.) we employed separately had almost no effect on HPA axis activity. In contrast, ethanol plus cyanamide treatment at these dose levels caused a dramatic increase in acetaldehyde concentration in this experiment with no peritoneal irritation.

Ethanol can easily cross the blood–brain barrier and has a wide range of actions in the central nervous system (Deitrich et al., 1989; Eckardt et al., 1998). Acetaldehyde is mainly formed in the liver by the process of ethanol oxidation. It is very difficult for acetaldehyde, derived from peripheral metabolism of ethanol, to penetrate from blood to brain. ALDH is widely distributed in brain (Zimakin, 1991) and in the microvasculature of the brain (Petersen, 1985) and it acts as a metabolic barrier to protect the brain from acetaldehyde. ALDH acts to eliminate acetaldehyde in blood as it enters the brain (Hunt, 1996). Very high levels (≥250 µM) of acetaldehyde in blood are needed before it can be detected in brain (Sipple, 1974), and elevated acetaldehyde is never detected in the brain of rats, even after high dose ethanol administration (Zimakin and Pronko, 1995). In this experiment, the combination of cyanamide and ethanol resulted in very high concentrations of acetaldehyde sufficient to penetrate the blood–brain barrier.

The mechanism and neurotransmitters involved in mediating the effects of acetaldehyde on CRF neurons in the PVN remain to be investigated, although dopamine, noradrenaline and 5-hydroxytryptamine have all been implicated (Truitt and Walsh, 1971; Brien and Loomis, 1983; Heap et al., 1995).

In conclusion, we have demonstrated that high concentrations of acetaldehyde generated by cyanamide, a potent inhibitor of ALDH, combined with a low dose of ethanol, resulted in increased plasma corticosterone concentrations and significant increases in both CRF mRNA and AVP mRNA in the PVN and POMC mRNA in the anterior pituitary. These results suggest that acetaldehyde is able to activate the HPA axis at a central level. Whether this activation is due to a direct action of acetaldehyde on CRF neurons in the PVN, or is due to modulation of other neurotransmitter systems activated in response to elevated acetaldehyde, remains to be determined.

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