EFFECTS OF ACETALDEHYDE ON c-fos mRNA INDUCTION IN THE PARAVENTRICULAR NUCLEUS FOLLOWING ETHANOL ADMINISTRATION

HIROSHI KINOSHITA*, DAVID S. JESSOP1, DAVID J. ROBERTS2, KIYOSHI AMENO3, IWAO IJIRI1, SHIGERU HISHIDA and MICHAEL S. HARBUZ1

Department of Legal Medicine, Hyogo College of Medicine, 1-1, Mukogawa-cho, Nishinomiya, Hyogo, 663-8501, Japan, 1URC for Neuroendocrinology, University of Bristol, BRI, Marlborough Street, Bristol BS2 8HW, 2School of Chemistry, University of Bristol, Cantocks Close, Bristol BS8 1TS, UK and 3Department of Forensic Medicine, Kagawa Medical University, 1750-1, Miki, Kagawa, 761-0793, Japan

(Received 8 November 2001; in revised form 5 February 2002; accepted 20 February 2002)

Abstract — Aims: The effect of acetaldehyde on c-fos mRNA expression in the paraventricular nucleus (PVN) of the rat was examined using in situ hybridization histochemistry. Methods: Increases in acetaldehyde concentrations were induced using cyanamide (a potent inhibitor of aldehyde dehydrogenase), in the presence of two different doses of ethanol. Concentrations of blood ethanol and acetaldehyde were determined by head space gas chromatography. Results: Neither cyanamide alone nor the low dose of ethanol (1 g/kg) alone increased c-fos expression in the PVN. However, the combination of cyanamide and low dose ethanol resulted in a significant and maximal increase in c-fos mRNA in the PVN. High dose ethanol (3 g/kg) resulted in a significant increase in c-fos mRNA. This stimulation also appeared maximal as there was no further increase in c-fos expression in the presence of cyanamide. Conclusions: These data suggest that acetaldehyde accumulation in blood has an important stimulatory effect on c-fos expression in the PVN at low ethanol concentrations. Furthermore, this stimulation of c-fos mRNA appears to be an either/or response: not activated in response to low dose ethanol, but maximally to high dose ethanol. These data also provide further evidence for a dissociation between the activation of c-fos and corticotrophin-releasing factor (CRF) mRNA in the PVN, as we have previously demonstrated that this dose of cyanamide alone is sufficient to evoke a sustained increase in plasma corticosterone and an increase in CRF mRNA.

INTRODUCTION

It has been reported that ethanol has many actions on neuroendocrine function and, in particular, that the hypothalamic–pituitary–adrenal (HPA) axis is activated by ethanol administration (Ellis, 1966; Rivier et al., 1984; Rivier and Vale, 1988; Thiagarajan et al., 1989; Rivier, 1996; Rivier and Lee, 1996; Ogilvie et al., 1997, 1998). The activation of the HPA axis occurs in response to stress and a variety of other challenges that activate the corticotrophin-releasing factor (CRF)-containing paraventricular nucleus (PVN) neurons that lead to increased CRF secretion. CRF stimulates adrenocorticotropic hormone (ACTH) secretion from the anterior lobe of the pituitary, and ACTH drives secretion of glucocorticoid from the adrenal gland (Harbuz and Lightman, 1997). Cyanamide, a potent inhibitor of aldehyde dehydrogenase (Deitrich et al., 1976), produces an accumulation of acetaldehyde when taken with ethanol (Sellers et al., 1981; Paechy and Naranjo, 1984). Acetaldehyde, the first metabolite of ethanol, appears to mediate some of the behavioural and central neurotoxic effects of ethanol, such as headache, palpitations, nausea and hypotension (Hunt, 1996; Eriksson, 2001), however, the details of its properties are still unclear (Brien and Loomis, 1983). We have reported that acetaldehyde is a potent stimulant of the HPA axis (Kinoshita et al., 2001a).

The expression of the immediate early genes (IEGs), such as c-fos, has been widely used as a marker of neuronal activity in the central nervous system (Sagar et al., 1988). Following ethanol (3 g/kg) administration, the levels of c-fos mRNA in PVN are markedly increased (Zoeller and Fletcher, 1994; Ogilvie et al., 1998). However, there have been no reports on the relationship between acetaldehyde and c-fos expression in the PVN. Our previous observations (Kinoshita et al., 2001a), prompted us to hypothesize that c-fos mRNA may be induced in the PVN by the accumulation of acetaldehyde in ethanol- and cyanamide-treated rats. In the present study, we examined the acute effects of acetaldehyde combined with ethanol on c-fos expression in the PVN using in situ hybridization histochemistry (ISHH).

MATERIALS AND METHODS

Adult male Sprague–Dawley rats weighing 220–250 g were used. They were housed individually in a temperature- and humidity-controlled environment and maintained on a 12-h light:12-h dark cycle. Food and water were available ad libitum. All experiments were started between 09:00 and 09:30. Rats were handled by the investigator daily to minimize the effects of handling in the experimental procedure.

Six experimental groups were used as follows: saline (as control), cyanamide (50 mg/kg) alone, low dose of ethanol (1 g/kg body weight) alone, high dose of ethanol (3 g/kg body weight) alone, low dose of ethanol with cyanamide and high dose of ethanol with cyanamide. Cyanamide (Sigma Chemical Co., Dorset, Poole, UK) or saline in a volume of 0.1 ml/100 g body weight was injected intraperitoneally (i.p.) 60 min before ethanol administration. Ethanol for i.p. injection was diluted with saline. Animals were killed by decapitation 30 min following ethanol administration and trunk blood was collected. This timepoint has previously been shown to be a suitable one for demonstrating a significant increase in c-fos mRNA after stress or ethanol administration (Imaki et al., 1992; Harbuz et al., 1993; Ogilvie et al., 1998). Brains were rapidly removed, frozen on dry-ice and stored at −80°C until sectioning. Sections 12 μm thick, containing the medial parvocellular region of PVN, were cut and thaw-mounted on gelatin-coated slides and stored at −80°C before hybridization. Trunk blood was collected for the measurement of ethanol and acetaldehyde levels. 

*Author to whom correspondence should be addressed.
Table 1. Concentration of acetaldehyde and ethanol in blood at 30 min after ethanol administration

<table>
<thead>
<tr>
<th>Group</th>
<th>Acetaldehyde (µM)</th>
<th>Ethanol (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol (1 g/kg)</td>
<td>8.1 ± 2.1</td>
<td>19.6 ± 1.5</td>
</tr>
<tr>
<td>Ethanol (1 g/kg) + cyanamide</td>
<td>392.8 ± 42.9*</td>
<td>21.4 ± 2.1</td>
</tr>
<tr>
<td>Ethanol (3 g/kg)</td>
<td>18.2 ± 2.3</td>
<td>64.5 ± 5.9</td>
</tr>
<tr>
<td>Ethanol (3 g/kg) + cyanamide</td>
<td>559.4 ± 35.1*</td>
<td>75.1 ± 5.4</td>
</tr>
</tbody>
</table>

Values are means ± SEM for n = 4 or 5 rats/group. *P < 0.001 vs matched dose of ethanol.

RESULTS

The concentrations of ethanol and acetaldehyde at 30 min following treatments are shown in Table 1. The administration of cyanamide had no significant effect on ethanol concentrations in either of the two ethanol-treated groups. In contrast, the acetaldehyde concentration in the ethanol with cyanamide-treated groups was markedly increased compared to the corresponding ethanol group in the absence of cyanamide [one-way ANOVA: F(3,12) = 143.40, P = 0.001 vs ethanol (1 g/kg) and vs ethanol (3 g/kg)]. Ethanol and acetaldehyde concentrations in control and cyanamide-treated rats were below the detection limits.

The changes in c-fos mRNA in the PVN, 30 min after ethanol administration, are shown in Fig. 1. These are expressed in arbitrary units. The control rats and those treated with cyanamide or ethanol (1 g/kg) alone exhibited very low levels of expression that were not significantly different. There was a significant (P < 0.05) increase in c-fos mRNA in the ethanol (1 g/kg) with cyanamide group [one-way ANOVA: F(5,19) = 5.22, P = 0.004] compared with the control, cyanamide alone and ethanol (1 g/kg) alone groups. Ethanol (3 g/kg) alone resulted in a significant (P < 0.01) increase in c-fos mRNA compared with the control, cyanamide alone and ethanol (1 g/kg) alone groups. Ethanol (3 g/kg) with cyanamide also resulted in a similar significant (P < 0.01) increase in c-fos mRNA. There were no significant differences comparing the high dose ethanol alone with the same dose given with cyanamide.

Gas chromatography

The concentration of ethanol and acetaldehyde was measured simultaneously by head-space gas chromatography (Okada and Mizoi, 1982).

Statistics

The data are expressed as mean ± SEM. All groups within each data set were compared by one-way analysis of variance (ANOVA) followed by Fisher’s protected least-significant difference test for multiple comparisons. A value of P < 0.05 was considered significant.

In situ hybridization histochemistry

ISHH was performed as described previously (Young et al., 1986; Harbuz and Lightman, 1989; Harbuz et al., 1991; Kinoshita et al., 2001a). In brief, the sections were warmed at room temperature, allowed to dry for 10 min, and then fixed in 0.25% acetic anhydride in 0.1 M triethanolamine/0.9% NaCl for 10 min. The sections were 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 for dehydration, delipidation and partial rehydration.

The probe used for c-fos was a 48-mer oligonucleotide complementary to part of the exonic mRNA sequence (Perkin–Elmer, Warrington, UK). The specificity of the probe has been determined previously (Harbuz et al., 1993). The probe was 3′-end-labelled with [35S]deoxy-ATP (1000 Ci/mmol; NEN, Boston, MA, USA) by terminal deoxynucleotidyl transferase (Boehringer–Mannheim, Lewes, Sussex, UK) and column-purified by QIA quick nucleotide removal kit (Qiagen Ltd, Crawley, West Sussex, UK). The specific activity of the probe was 6.70 × 108 d.p.m./mg. Approximately 100 000 c.p.m. probe (per 45 µl) were applied to each slide. Hybridization was performed overnight at 37°C. All the sections were processed 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) using a computer-assisted image analysis system (Image 1.22, developed by W. Rasband, NIH, Bethesda, MD, USA) run on Apple Macintosh.

Fig. 1. c-fos mRNA levels in the paraventricular nucleus of rats. Levels were determined following administration of saline (CONTROL), cyanamide (50 mg/kg; CY), ethanol (1 g/kg; E1), ethanol (3 g/kg; E3), ethanol (1 g/kg) with cyanamide (CY+E1) and ethanol (3 g/kg) with cyanamide (CY+E3). These are expressed in arbitrary units. Values are presented as mean ± SEM (n = 4 or 5). *P < 0.05 compared with CONTROL, CY and E1: **P < 0.01 compared with CONTROL, CY and E1.
DISCUSSION

The expression of c-fos in the PVN was quantitatively evaluated by ISHH, and blood ethanol and acetaldehyde concentrations were also determined. Acetaldehyde stimulated c-fos expression in response to an anxiolytic dose (Eckardt et al., 1998) of ethanol (1 g/kg) combined with cyanamide, compared to ethanol (1 g/kg) alone or cyanamide alone, which had little effect on c-fos mRNA induction. However, in response to the high dose of ethanol (3 g/kg) alone, induction of c-fos gene expression was observed in the present work in PVN similarly to the results of previous studies (Zoeller and Fletcher, 1994; Ogilvie et al., 1997). As the high dose of ethanol itself had a potent stimulatory effect on c-fos mRNA, the effect of accumulated acetaldehyde was completely masked by the high dose of ethanol, although higher acetaldehyde concentration was observed in ethanol (3 g/kg) with cyanamide-treated rats. These data suggest that this represents a maximal effect on c-fos expression which does not occur with low dose ethanol alone, but does occur with either ethanol + cyanamide or a high dose of ethanol. There has been a report of c-fos induction in fat-storing cell cultures by acetaldehyde (Casini et al., 1994). This study presents the first evidence that acetaldehyde can cause an increased c-fos gene expression in the PVN.

The expression of c-fos is rapidly induced in various brain regions in response to a variety of physiological manipulations, such as immobilization, hypertonic saline injection, irradiation, cocaine or morphine administration and high dose ethanol administration (Cecchetti et al., 1989; Imaki et al., 1992; Moratalla et al., 1993; Liu et al., 1994; Zoeller and Fletcher, 1994; Usenius et al., 1996; Ogilvie et al., 1998). However, the role of c-fos in the central nervous system is unclear; it has been proposed that IEGs, including c-fos, have a function as mediators of the response to external stimuli (Sheng and Greenberg, 1990). Acute stress results in an increase in c-fos mRNA in CRF-containing neurons in the PVN, suggesting a close relationship (Cecchetti et al., 1989; Imaki et al., 1992). The activation of c-fos and CRF gene expression have been linked following ethanol administration (Ogilvie et al., 1998). However, the precise relationship between CRF and c-fos in the PVN remains obscure and has been the subject of much investigation, resulting in a number of studies that have questioned this relationship. The induction of heteronuclear CRF mRNA has been observed prior to an increase in c-fos mRNA, suggesting that Fos protein may not mediate the activation of CRF gene transcription (Kovacs and Sawchenko, 1996). It has also been noted that c-fos mRNA in the PVN can be dissociated from the activation of CRF mRNA in the rat model of adjuvant-induced arthritis (Harbuz and Jessop, 1999). In addition, the CRF promoter lacks an activator protein (AP)-1 site (Ogilvie et al., 1998). In our previous study (Kinoshita et al., 2000), we noted a significant and sustained increase in plasma corticosterone that was maintained for 4 h and that was reflected by an increase in CRF mRNA in the parvocellular cells of the PVN following this dose of cyanamide alone. The ability to increase CRF mRNA (Kinoshita et al., 2000) in the absence of a change in c-fos mRNA (the present study) provides further evidence for a dissociation between the activation of c-fos and CRF mRNA. Taken together, these data question the relationship between CRF and c-fos. However, although accumulating evidence suggests c-fos mRNA does not appear to regulate directly CRF mRNA expression, it has been suggested that Fos protein may interact with other transcription factors to modulate CRF gene expression (Imaki et al., 1996), and fos remains a useful marker of neuronal activation.

In the present study, we could not determine whether acetaldehyde activates PVN neurons in a direct or indirect manner. The c-fos mRNA activation in fat-storing cell cultures may be mediated by protein kinase C activation (Casini et al., 1994). However, as both CRF and c-fos promoters have a functional cyclic AMP (cAMP) response element, it has been speculated that a cAMP-dependent system might mediate this activation (Kovacs and Sawchenko, 1996; Ogilvie et al., 1998). The relationship between CRF neurons and c-fos in the PVN remains to be determined, but may involve other neurotransmitter systems. It has been reported that glutamate is a major neurotransmitter in the region of the PVN (Bramm, 1995, and glutamate, catecholamine and acetylcholine are candidate neurotransmitters for mediation of the induction of a number of IEGs including c-fos in the PVN (Imaki et al., 1996). Acetaldehyde modulates monoamine metabolism in brain (Hashimoto et al., 1989) and can induce catecholamine release from peripheral adrenergic nerve terminals (Brien and Loomis, 1983; Chiba and Tsukada, 1988). Also in the periphery, high concentrations of acetaldehyde have been noted to stimulate cholinergic neurons in intestine (Kinoshita et al., 2001b). However, there are no reports describing the effects of acetaldehyde on the release of neurotransmitters in PVN and the involvement of these requires further investigation. A number of studies have investigated the interaction of the cAMP response element and the glucocorticoid response element in the CRF gene. Clearly, glucocorticoid feedback (via the type II glucocorticoid receptor present in the PVN), is a potent regulator of CRF mRNA. Future studies could elucidate the interplay of these elements and neurotransmitters following treatment with acetaldehyde.

In conclusion, we have demonstrated that acetaldehyde significantly and maximally increased c-fos mRNA in the PVN in the presence of a low dose of ethanol. These results suggest that acetaldehyde exerts central actions inducing neuronal activation, resulting in activation of the HPA axis. These data also provide further evidence for a dissociation between the activation of c-fos mRNA and CRF mRNA in the parvocellular cells of the PVN. It remains to be determined whether this activation is due to a direct action of acetaldehyde on CRF neurons, or due to modulation of other neurotransmitter systems activated in response to elevated blood concentrations of acetaldehyde.

Acknowledgements — This work was partially supported by a grant from the Daiwa Anglo-Japanese Foundation, Asahi Breweries Foundation and a grant-in-aid for Encouragement of Young Scientists, No. 13770224 from the Japanese Society for the Promotion of Science. We are grateful to Drs D. P. Finn and T. L. Coventry for their technical assistance.

REFERENCES


