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Distribution and Differential Induction of CYP2E1 by Ethanol and Acetone in the Mesocorticolimbic System of Rat

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Abstract — Aims: The expression of cytochrome P4502E1 (CYP2E1) in the brain has been demonstrated in several regions, nevertheless there is a lack of specific studies on the constitutive expression and induction at the mesocorticolimbic system, the most relevant brain pathway in the context of drug addiction and alcoholism. Hence, we have performed a detailed study of the CYP2E1 expression and induction in three key areas of the mesocorticolimbic system of the rat brain: prefrontal cortex (PFC), nucleus accumbens (NAc), and ventral tegmental area (VTA). Methods: Expression levels of CYP2E1 were analyzed by Western blot. The induction of the enzyme in the selected brain areas by chronic acetone (1% v/v acetone in drinking water for 11 days) and ethanol (3 g/kg by gavage for 7 days) was also assessed. Results: (i) CYP2E1 was expressed in PFC, Nac, and VTA, with the order of magnitude of the levels being VTA ~ PFC > Nac, and approximately 3–13% of it was encountered in liver; (ii) acetone treatment significantly increased CYP2E1 expression in Nac, up to 212% of the control levels, whereas not significant changes were observed in VTA and PFC; (iii) chronic ethanol treatment only resulted in a significant induction of enzyme levels in VTA (124%). A similar enhancement, though not significant, was found to occur in Nac. Conclusions: CYP2E1 was present in the mesocorticolimbic system at different levels of expression. Chronic acetone and ethanol treatments are able to increase enzyme levels in specific areas of this system with the pattern of induction of the two agents being different.

INTRODUCTION

A vast amount of evidence has emerged suggesting that both natural rewards and drugs of abuse, including ethanol, derive their reinforcing properties by acting through a common pathway involving the brain neurotransmitter dopamine (DA) (Wise and Rompre, 1989; Hyman and Malenka, 2001; Koob and Le Moal, 2006). Concretely, the reinforcing effects of ethanol have been found to be associated with several brain structures belonging to the mesocorticolimbic dopamine system. In this system, dopamine neurons originate in the ventral tegmental area (VTA) of the brain stem and innervate the prefrontal cortex (PFC), nucleus accumbens (NAc), and ventral tegmental area (VTA). Methods: Expression levels of CYP2E1 were analyzed by Western blot. The induction of the enzyme in the selected brain areas by chronic acetone (1% v/v acetone in drinking water for 11 days) and ethanol (3 g/kg by gavage for 7 days) was also assessed. Results: (i) CYP2E1 was expressed in PFC, Nac, and VTA, with the order of magnitude of the levels being VTA ~ PFC > Nac, and approximately 3–13% of it was encountered in liver; (ii) acetone treatment significantly increased CYP2E1 expression in Nac, up to 212% of the control levels, whereas not significant changes were observed in VTA and PFC; (iii) chronic ethanol treatment only resulted in a significant induction of enzyme levels in VTA (124%). A similar enhancement, though not significant, was found to occur in Nac. Conclusions: CYP2E1 was present in the mesocorticolimbic system at different levels of expression. Chronic acetone and ethanol treatments are able to increase enzyme levels in specific areas of this system with the pattern of induction of the two agents being different.

The enzymatic machinery necessary to metabolize ethanol has been reported to be present in the brain, consisting of several enzymes of which catalase and cytochrome P4502E1 (CYP2E1) are particularly relevant (Zimatin et al., 2006). In relation to CYP2E1, the major ethanol-inducible CYP, the identification of its constitutive expression in the brain as well as its regional distribution has been extensively investigated in the last and present decade employing very different methodologies (Tindberg and Ingelman-Sundberg, 1996; Upadhya et al., 2000; Kapoor et al., 2006). Although, the expression of this enzyme in the brain is clearly region- and cell-specific (Hipólito et al., 2007), discrepancies exist regarding its precise distribution within the brain. Similarly, though all the studies demonstrate that brain enzyme levels are significantly lower than those observed in liver, a debate exists in relation to the precise levels of enzyme expression. Thus, some reports indicate extremely low levels of expression in the brain (Hansson et al., 1990; Roberts et al., 1994; Warner and Gustafsson, 1994; Montoliu et al., 1995; Tindberg and Ingelman-Sundberg, 1996; Yadav et al., 2006) whereas other studies show CYP2E1 levels to be 25% of liver levels (Anandatheerthavarada et al., 1993; Upadhya et al., 2000).

Moreover, it is surprising that there is a lack of detailed and specific studies on the constitutive expression at the mesocorticolimbic system, which is probably, as we have commented above, the most relevant brain pathway in the context of drug addiction and alcoholism. The small amount of data reported, using immunochemistry methods, show CYP2E1 immunoreactivity in the rat nucleus accumbens (Hansson et al., 1990; Joshi and Tyndale, 2006) and in the cytoplasm of cells in the VTA and pars compacta of substantia nigra (SN) (no in pars reticulata) (Riedl et al., 1996). These data do not always agree; for example, Hansson et al. (1990) showed a high intensity of staining in NAc of rat brain whereas, more recently, Joshi and Tyndale (2006) reported weak immunocytochemical staining in NAc of monkey brain. Surprisingly, other authors (Yadav et al., 2006) were unable to detect CYP2E1 immunoreactivity, by Western blot, in the striatum and in the cortex. Additionally, to our knowledge, the expression levels of CYP2E1 in relevant
areas such as nucleus accumbens (NAc) and ventral tegmental area (VTA) have never been measured by immunoblotting.

On the other hand, although the pattern of CYP2E1 induction by ethanol in the brain has been widely studied by using different methodologies and different protocols of induction, some contradictory data have been reported (see Hipólito et al., 2007, for review). Globally, it can be said that the results are consistent with CYP2E1 ethanol induction in the brain, which is highly localized in certain regions. Nevertheless, once again scant or null data are reported on the enzyme induction in the mesocorticolimbic dopamine system. Concretely, the potential induction in NAc and VTA has never been quantified by Western blot. In PFC, some immunoblotting data have shown enzyme induction (Upadhya et al., 2000; Howard et al., 2003) whereas other studies failed to find induction (Yadav et al., 2006). In our opinion, the knowledge of CYP2E1 expression and induction in the mesocorticolimbic system could be of special interest since localized enzyme induction in certain zones such as PFC, VTA, and NAc could be sufficient to substantially alter local metabolism of ethanol hence increasing acetaldehyde concentrations. This elevated metabolite levels together with the high levels of DA observed in these areas (Gonzales et al., 2004) might, then, result in increased concentrations of isoquinoline derivatives, which could participate in the neuropharmacological effects of ethanol. Some animal and human studies indirectly suggest that this hypothesis could be plausible. For example, it has been found that salsolinol, the condensation product of acetaldehyde with dopamine, is elevated in the brain of alcoholics and rats chronically treated with ethanol (Sjoquist et al., 1982a; Sjoquist et al., 1982b; Haber et al., 1996). Additionally, some authors have reported that salsolinol has rewarding properties in NAc of rats (Rodd et al., 2003).

Consequently, to shed light on this topic, we have explored CYP2E1 expression in the prefrontal cortex, nucleus accumbens, and ventral tegmental area, measuring their levels by Western blot analysis and comparing the effects of acetone and ethanol—the two recognized inducers of this cytochrome (Yang et al., 1991; Forkert et al., 1991; Koop, 1992)—on the enzyme levels. The study provides the first known documentation of enhanced CYP2E1 levels in NAc caused by acetone treatment in addition to a significant induction of the enzyme in VTA and an increase of levels in NAc after chronic ethanol treatment.

MATERIALS AND METHODS

Animals

Male Wistar rats (300–330 g) were accommodated in a temperature-controlled (23 ± 1°C) room and maintained in a 12 h light/dark cycle (on 08:00, off 20:00). Standard rat chow and water were made available ad libitum. Wistar rats were chosen for this study since they have been used in the majority of the reported data that studied CYP2E1 in the brain (Anandatheerthavarada et al., 1993; Howard et al., 2003; Yadav et al., 2006). All procedures described in the present study were conducted in accordance with the EEC Council Directive 86/609, the Spanish laws (RD 223/1988), and policies on protection of animals, and were approved by the Animal Care Committee of the University of Valencia.

Acetone and ethanol treatment

Two different induction protocols were compared in the present study. The first used acetone as inducer and was adapted from Forkert et al. (1991). In this protocol rats were administered acetone (1%, v/v) in drinking water for 11 days (n = 6). Acetone was omitted from the drink water of control rats (n = 6). Acetone solutions were daily prepared and replaced. Animals were housed individually, thus the daily volume ingested and the weight of animals were measured. During the three first days, the rats belonging to the acetone-treated group drank approximately half the volume that control rats drank—35 ml per day against 60 ml per day. But at the end of the treatment, the volumes ingested were very similar (45 ml per day for the treated rats against 50 ml per day for the control group). Hence, in our experimental conditions, the addition of acetone in the drinking water does not affect the pattern of drinking. Similarly, the increase in the weight of animals was around 30 g in both the control and treated groups.

The second protocol used ethanol and is the same than that reported by Howard et al. (2003). In this case, rats were housed individually and ethanol at 3.0 g/kg (n = 6) or saline (n = 6) was given once a day for 7 days by gavage. This dose of ethanol has been proven to produce observable CNS effects, including sedation and motor impairment (Le and Israel, 1994).

Brain dissection and protein extraction

The last day of the acetone treatment or after 3 hours of the last dose of ethanol, animals received a lethal dose of chloral hydrate by i.p. route, and brains were quickly removed and frozen in isopentane. Serial frontal sections (40 μm) were cut on a cryostat, achieving the brain area to be studied. The A/P stereotaxic coordinates of each area (PFC, Nac, and VTA) were established previously according to the atlas of Paxinos and Watson (Paxinos and Watson, 1986). Samples were obtained by punching a portion from an approximately 1-mm-thick coronal slice that included the tested areas. Each of the portions obtained were, then, homogenized in RIPA buffer (phosphate-buffered saline containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)), and protease inhibitors. Thereafter, the homogenates were centrifuged at 13,200 × g for 10 min at 4°C to eliminate large cells debris. The supernatant was used as total protein sample. Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, Madrid, Spain). This procedure was adapted from a procedure that we used previously (Montoliu et al., 1994) in all the animals liver was also removed. A piece of this organ was frozen, kept at −24°C, and posteriorly prepared for immunoblotting as described above for brain samples.

Western blotting

Proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) gels (4.5% acrylamide stacking gel and 12% acrylamide resolving gel), transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Billerica, MA, USA) using a mini trans-blot apparatus (MiniProteinII, Bio-Rad) for an hour. Membranes were then blocked in 5% nonfat dried milk in TBS and incubated overnight at
4°C with the primary antibody: anti-CYP4502E1 monoclonal antibody (1:4000; Oxford Biochemical Research, Barcelona, Spain). After three washes in TBS containing 0.1% Tween-20 (TBST), the blots were incubated for 1 hour with horseradish peroxidase–conjugated secondary antibody (1:10000;Santa Cruz Biotechnology, Madrid, Spain). Finally, blots were developed using the enhanced chemiluminescence system (ECL Plus, Amersham Bioscience, Little Chalfont, UK) according to manufacturer’s protocol. Homogeneity in the amount of protein loaded was confirmed by running separate gels with the same amount of protein from liver samples and, subsequently, by Coomassie Brilliant Blue gel staining (coefficient of variation was less than 5%). Digital images of the immunoblots were analyzed using the Quantity One Quantification Software version 4.6.1 (Bio-Rad, USA). The intensity of the bands was expressed as arbitrary units. The acetone- and ethanol-treated groups were determined by setting the control group to 100% and calculating the respective percentages, expressed as mean ± SEM.

Previously, untreated rat samples from each brain region tested were serially diluted separately and used to construct standard curves in order to determine the linear range of detection for immunoblotting assay. Material from three or four rats was pooled for this analysis. According to the results obtained, the following amounts of proteins were loaded: 20 µg of PFC, 40 µg of Nac, and 30 µg of VTA.

Statistical analysis

The differences among expression levels of 20 µg of protein in the three brain areas were tested by one-way analysis of variance (ANOVA) followed by a post hoc test (Scheffé test). Differences in enzyme levels between the studied brains area of control and treated rats were analyzed using unpaired Student’s t-test. This test was applied to data obtained in PFC, Nac, and VTA. The level of significance was set at P < 0.05 for all comparisons.

RESULTS AND DISCUSSION

Detection of CYP2E1 in mesocorticolimbic areas of rat brain: Levels of expression

An immunoblotting assay was developed to detect and measure CYP2E1 in PFC, Nac, and VTA. The immunoreactive band from brain samples comigrated with a band from untreated rat liver samples (Fig. 1A) and close to the band from a prestained marker of ~50 kDa (Chemicon® Western Control, Sigma, Missouri, USA) (Fig. 1B). A dilution curve of proteins of each brain region of untreated rats was constructed. The linear range of detection was different in the three areas analyzed, indicating differences in the expression levels. For example, the standard curve of PFC samples is shown in Fig. 1C. As can be seen, the CYP2E1-reactive signal was linear from 10 to 80 µg of protein. In the case of Nac and VTA, the linear range oscillates from 20 to 100 µg and from 10 to 40 µg, respectively. These results put in evidence the validity of the quantification method and, at the same time, can be used to compare the expression levels in the three studied areas. Fig. 2 shows the CYP2E1 levels in 20 µg of protein from PFC, Nac, and VTA samples. These values were taken from the standard curves. The one-way ANOVA analysis revealed that the expression levels were statistically different in the three regions (F(2,5) = 0.13, P = 0.013). The post hoc analysis (Scheffé test) showed that the levels in Nac were significantly lower (approximately fourfold) than those found in PFC (P < 0.05) and VTA (P < 0.05) and in these two areas no significant differences between enzyme levels were found. Recently, Howard et al. (2003) measured, by immunoblotting,
rat CYP2E1 levels in several brain areas including frontal cortex and striatum. They found that the striatum was the region with the lowest amount of enzyme, being the levels in frontal cortex quite twofold higher than in striatum. Since the NAc is a part of the ventral striatum, it could be said that these findings agree with our results and confirm the low CYP2E1 levels in the striatum, in general, and in the rat NAc in particular, compared to others regions of the mesocorticolimbic system.

Furthermore, our immunoblotting data from liver samples show that CYP2E1 expression in the mesocorticolimbic areas studied is really low with respect to that observed in the liver. Concretely, the enzyme levels in PFC, Nac, and VTA have been shown to be, approximately, 8%, 3%, and 13% of the liver levels, respectively.

To our knowledge, this is the first detailed report of the distribution of CYP2E1 among several areas of the mesocorticolimbic system of rat brain. It is also the first time that CYP2E1 expression in NAc and VTA is firmly demonstrated and, what is more important, our data clearly show that different enzyme levels exist in the areas studied, which may play an
important role in ethanol metabolism in the mesocorticolimbic system.

**Differential induction of CYP2E1 in mesocorticolimbic areas of rat brain: Comparison between chronic acetone and ethanol treatments**

The effect of chronic ethanol and acetone treatment on CYP2E1 expression levels in the liver had been previously compared, showing a different pattern of induction (Koop et al., 1985; Forkert et al., 1991). However, no similar studies have been performed in the brain. As both substances can be used as pharmacological tools to induce brain CYP2E1, we planned our experiments in order to investigate and compare the effect of both inducers in the CYP2E1 expression levels in the mesocorticolimbic system.

Using the sensitive immunoblotting assay described above, we have found that acetone treatment enhanced the CYP2E1
expression in the liver significantly, with almost threefold difference between the levels in the control and the treated group (Fig. 3). Consequently, the efficacy of acetone treatment was proved. Our results are in complete accordance with those described previously by Roberts et al. (1994) who, using a similar acetone-induction protocol and immunoblotting analysis, found in the liver a threefold increase in CYP2E1 compared to controls.

In our experimental conditions and in the brain areas studied, the induction of CYP2E1 by acetone treatment was region-selective. Hence, treatment was only able to significantly induce CYP2E1 levels in the NAc up to 212% of the control levels \((P < 0.0001)\). On the contrary, PFC and VTA enzyme levels were not modified significantly (Fig. 3).

On the other hand, the ethanol-induction protocol was effective. So, treatment with a behaviorally relevant dose of ethanol (3 g/kg) for 7 days significantly increased liver CYP2E1 up to 157% of the control value (Fig. 4). This enhancement was lower than that observed for acetone treatment. Acetone and ethanol are two recognized inducers of hepatic CYP2E1, with acetone being the more potent inducer (Koop et al., 1985; Forkert et al., 1991), which is in agreement with the results we obtained in the liver. However, as commented above, little is known about their comparative effects on CYP2E1 expression levels in the mesocorticolimbic system. According to our data, the induction by ethanol treatment was region specific, as occurred with acetone, though a different pattern of induction was observed (Fig. 4). Curiously, after chronic ethanol treatment, a significant enhancement was observed in the enzyme levels in VTA: \(124.69 \pm 5.9\%\) of control levels. In NAc a similar increase in induction was observed \((124.79 \pm 10.1\%\) of control levels) although no statistically significant differences were detected, probably due to the higher variability. This difference in the profile of induction between acetone and ethanol may be attributed to differences in the specific induction mechanism and also may represent differences in ethanol and acetone distribution in the areas analyzed.

The evidence of enhanced CYP2E1 expression in two key areas (VTA and NAc) of the mesolimbic dopamine system after chronic use of ethanol, measured by Western blot, is reported for the first time. The localized CYP2E1 induction may be of significance because enzyme levels are increased in brain areas where ethanol is thought to exert some of its neurobiological and behavioral effects (Koob and Le Moal, 2006).

Our results also show that, as occurs in the case of acetone treatment, CYP2E1 levels in the PFC are not induced by ethanol. The data reported in the literature, concerning the enzyme induction by ethanol in the PFC measured by Western blot, are scarce and do not always agree. For example, Upadhyia et al. (2000) showed a significant increase in the brain cortex after a chronic ethanol treatment (ethanol 10% v/v in sucrose solution (1%, w/v) for 30 days), whereas recently Yadav et al. (2006) did not find induction of CYP2E1 in rat brain cortex after the administration of a single dose of ethanol (0.8 ml/kg body weight, i.p.). Surprisingly, Howard et al. (2003), using the protocol we have used and a polyclonal CYP2E1 antibody, showed a twofold increase in the frontal cortex of rats. Although in all these studies, as well as in our work, immunoblotting technique and the rats were used to measure CYP2E1 levels, the differences in the ethanol treatment, specific antibody and brain samples preparation could be responsible of the observed discrepancies.

In summary, CYP2E1 was found to be present in the mesocorticolimbic system at different levels of expression. Its expression can be induced in NAc by acetone treatment and in VTA and NAc by ethanol treatment. Therefore, our data imply that acetaldehyde production by CYP2E1 in these brain areas could be higher in chronic ethanol abusers, making it probable that this metabolite or its condensation products with DA contribute to the pathophysiology of alcoholism.

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