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Effects of ethanol on extracellular levels of adenosine in the basal forebrain: An *in vivo* microdialysis study in freely behaving rats

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Abstract

BACKGROUND—Adenosine is implicated to play a pivotal role in mediating many neuronal responses to ethanol. While *in vitro* studies performed in cell culture have demonstrated that acute ethanol exposure increases extracellular adenosine levels, this effect has not been demonstrated, *in vivo*, in the brain. We performed an *in vivo* microdialysis study to examine the effects of local ethanol perfusion on extracellular levels of adenosine in the basal forebrain.

METHODS—Under sterile conditions and using a standard surgical protocol, adult male Sprague-Dawley rats were implanted with unilateral microdialysis guide cannula targeted towards the basal forebrain. Following post-operative recovery, the microdialysis probe was inserted. After allowing at least 12–16 hr for probe insertion recovery, the experiment was begun. Artificial cerebrospinal fluid was perfused (0.7 μ L/min) for 80 min and 4 $\times$ 20 min pre-ethanol baseline samples were collected. Subsequently, 30, 100 and 300mM doses of ethanol were perfused. Each ethanol dose was perfused for 80 min and 4 $\times$ 20 min samples were collected. Finally, aCSF was perfused and 4 $\times$ 20 post-ethanol samples were collected. Adenosine in the microdialysate was separated and measured with HPLC coupled with an UV detector. On completion, the animals were euthanized, brain removed and processed for histology.

RESULTS—Local ethanol perfusion in the basal forebrain produced a significant increase in extracellular adenosine with the highest dose of 300 mM ethanol producing a 4 fold increase. Cresyl violet (Nissl) staining did not indicate any toxic damage in the area surrounding the probe tip. Choline acetyltransferase immunohistochemistry revealed that all microdialysis probe sites were localized in the basal forebrain.

CONCLUSION—Our study is the first to demonstrate that ethanol acts directly in the brain to increase extracellular adenosine.

Keywords

Adenosine; Basal Forebrain; Ethanol; Microdialysis; Cholinergic

INTRODUCTION

Consistent evidence exists to suggest that the neuromodulator adenosine has a direct role in mediating many cellular and behavioral responses to ethanol including ataxia, anxiety, seizures and tremors (Dunwiddie and Masino 2001; Hack and Christie 2003; Mailliar and Diamond 2004; Newton and Messing 2006). Intracerebellar and intrastriatal administration of

adenosinergic agonists significantly and dose-dependently accentuated ethanol induced ataxia. In contrast, adenosine antagonists significantly and dose-dependently attenuated ethanol induced ataxia (Meng and Dar 1995; Dar and Mustafa 2002; Dar 2002; Dar 1997). Systemic administration of adenosine and its receptor agonist reduced multiple signs of ethanol withdrawal including tremors, anxiety and anxiogenic-like responses (Batista *et al.* 2005; Kaplan *et al.* 1999; Prediger *et al.* 2006). Chronic ethanol treatment increased A₁ receptor density in the cortex and reduced S - (4-nitrobenzyl)-6-thioinosine (NBTI) sensitive type 1 equilibrative nucleoside transporter-1 (ENT1) in striatum (Kaplan *et al.* 1999; Jarvis and Becker 1998).

Mice with constitutive knockout of ENT1 gene displayed decreased adenosinergic tone resulting in increased ethanol consumption coupled with reduced hypnotic (loss of righting reflex) and ataxic responses to ethanol. While treatment with an A₁ receptor agonist locally in the nucleus accumbens decreased ethanol consumption in ENT1-null mice, A_{2A} receptor agonist was ineffective (Choi *et al.* 2004). Mice with constitutive knockout of the adenosine A_{2A} receptor showed increased ethanol consumption and reduced hypnotic response to ethanol (Naassila *et al.* 2002). A recent study showed that transgenic mice that express human ENT1 in neurons displayed increased sensitivity to ethanol (Parkinson *et al.* 2009). While the above studies implicate adenosine as a mediator of ethanol's effects, the source of adenosine in the brain is unknown.

Three mechanisms have been proposed to explain the interaction between ethanol and adenosine (Mailliard and Diamond 2004). The first proposed mechanism is based on the studies performed in cell cultures and suggests that ethanol may block the reuptake of adenosine by inhibiting ENT1 activity (Nagy *et al.* 1990; Krauss *et al.* 1993). The second mechanism proposes that ethanol metabolism in the liver results in adenosine release in blood circulation that can freely cross the blood-brain barrier and increase extracellular adenosine in the brain (Orrego *et al.* 1988; Carmichael *et al.* 1991; Carmichael *et al.* 1993; Cornford and Oldendorf, 1975). The third mechanism proposes that ethanol metabolism in the liver results in the release of acetate in the blood that can freely cross the blood brain barrier. Once in the brain, acetate is readily converted into adenosine (Berl and Frigyesi 1969).

We hypothesized that ethanol will act directly in the brain to increase extracellular adenosine. We tested our hypothesis by performing an *in vivo* microdialysis study to examine the effects of ethanol on extracellular levels of adenosine. Since ethanol metabolism in the periphery can indirectly increase adenosine levels in the brain, we performed local reverse microdialysis administration of ethanol to verify the direct effects.

METHODS

Every effort was made to minimize animal suffering and to reduce the number of animals used. All animals were treated in accordance with the American Association for Accreditation of Laboratory Animal Care's policy on care and use of laboratory animals. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and all experimental protocols were approved by the Animals Committee of the Harry S. Truman Memorial Veterans Hospital.

Animals & Surgery

Adult male Sprague-Dawley rats (250–350g) were housed under constant temperature, with *ad libitum* access to food and water, and with 12-h light/dark cycle (Lights-On at 2300 hr and Lights-Off at 1100 hr) for at least 10 days before surgery. Under sterile conditions and using a standard surgical protocol [for details see (Thakkar *et al.* 2003b)] a microdialysis guide cannula (Eicom Inc, San Diego CA) was implanted unilaterally at 90° angle above the basal

forebrain (BF) [target coordinates for the tip of the microdialysis probe were posterior 0.3, lateral 2.5, ventral 9.0: relative to bregma and skull surface at bregma (Paxinos and Watson 2007)] along with two anchor screws and a dummy plug (MS363, PlasticsOne, Roanoke, VA). The side (left or right BF) of guide implantation was counterbalanced to avoid any side (left vs right BF) effects. The entire assembly was secured to the brain with dental cement.

Ethanol

The 200 proof absolute ethyl alcohol (Acros Organics) was purchased from Fischer Scientific. Subsequent dilutions were made fresh in artificial cerebrospinal fluid (aCSF = NaCl 147 mM, KCl 3 mM, CaCl₂ 1.2 mM, MgCl₂ 1.0 mM, pH 7.2). All salt used for preparation of aCSF were purchased from Fisher Scientific.

Ethanol perfusion and microdialysis sampling

Experiments were conducted in a sound-attenuated chamber with the same light conditions as described above. Food and water was available *ad libitum*. After at least 5 days of post-operative recovery, a microdialysis probe (CX-1-8-02, 2 mm membrane length, 0.22 mm O.D; Eicom, San Diego, CA) was inserted in the guide cannula as follows: The rat was gently swaddled in a towel. The stylus was removed. The microdialysis probe was gently inserted into guide cannula. A cable (one end, Model 363-SL/6, PlasticOne, Roanoke, VA) was connected to the dummy plug to support the microdialysis lines. After connecting the microdialysis inline and outline to the probe, the flow through the microdialysis probe was verified. Next the cable (other end) was connected to a swivel (Model SL6C, PlasticsOne, Roanoke, VA). The inline was connected to a microdialysis pump (Model KDS250, KD Scientific, Holliston, MA) and the outline was connected to a collection vial. Subsequently, aCSF perfusion was begun with a flow rate of 0.7 µl/min. After allowing at least 12–16 hr of probe insertion recovery and for equilibrium at the probe tip, the experiment was begun at dark onset and 4 × 20 min (14 µL/sample) pre-ethanol baseline samples were collected. Subsequently, 30, 100 and 300mM doses of ethanol were perfused. Each dose of ethanol was perfused for 80 min and 4 × 20 min samples were collected. Finally, aCSF was perfused and 4 × 20 post-ethanol samples were collected. The flow rate was maintained at 0.7 µl/min during the entire experiment. The samples were stored in ice until analyzed.

Measurement of Extracellular Adenosine

Adenosine separation and quantification was performed by high performance liquid chromatography (HPLC) couple with UV detector, using the principle of liquid chromatography. This method has been extensively used by our laboratory and others to measure adenosine levels from basal forebrain (Porkka-Heiskanen et al., 1997; McKenna et al., 2007; Kalinchuk et al., 2008; Murillo-Rodriguez et al., 2004; Blanco-Centurion et al., 2006; Murillo-Rodriguez et al., 2008). 10 µl of microdialysis sample were injected into the HPLC. The sample was carried through the system in mobile phase containing 8 mM NaH₂PO₄ and 8% methanol (pH = 4) at a flow rate of 80 µl/min (see Porkka-Heiskanen *et al.* 1997; McKenna *et al.* 2007 for details). Adenosine was separated out with a microbore column (1 × 100 mm; MF-8949; BASi, West Lafayette, IN) and detected by a UV detector (Model SPD20, Shimadzu Scientific Instruments, Columbia MD) at 258 nm wavelength. The chromatogram data was acquired and analyzed by PowerChrom 280 system (eDAQ Inc, Colorado Springs, CO). Adenosine peak in the sample was identified and quantified by comparing its retention time and area under the peak to pure known amounts of external adenosine standards (Sigma, St. Louis MO).

Histology and Immunohistochemistry

On completion of the experiment, the microdialysis probe was removed and used to calculate *in vitro* recovery. The animal was euthanized under deep phenobarbital anesthesia and perfused transcardially with 200 mL of chilled 0.9% saline followed by perfusion with 200 mL of chilled 10% formalin (Sigma, St. Louis, MO). The brain were removed, blocked and processed for histology and immunohistochemistry (Thakkar *et al.* 2008). The blocked brain containing the BF region was sectioned and three series of 40 μ m sections were obtained. One series was used to perform cresyl violet staining as described previously (Thakkar *et al.* 2002). The cresyl violet staining was performed to assess any potential neuronal damage due to ethanol perfusion. The second series was used to perform choline acetyltransferase (ChAT) immunohistochemistry to localize the microdialysis sites within the cholinergic BF as described previously (Thakkar *et al.* 2001).

In vitro recovery

Each dialysisprobe, removed from the animal, was placed into a known concentration of adenosine and the amount of adenosine (aCSF flow rate =0.7 μ L/min) recovered by the probe was quantified.

Statistical Analysis

One way repeated measure ANOVA followed by the Bonferroni's post hoc test was performed to evaluate the effect of ethanol on adenosine release.

RESULTS

Out of a total of 10 rats, the microdialysis probe malfunctioned during the experiment in one rat and the probe site was a "mishit" and therefore, the data from those two animals was excluded.

Histology

The localization of microdialysis tips for each of the animals is shown in a coronal schematic Figure 1 Panel A (N=8, adapted from Paxinos and Watson 2007). All microdialysis sites were in the cholinergic BF between antero-posterior levels -0.3 and -0.6. A representative photomicrograph illustrating the microdialysis probe site in the cholinergic BF is shown in Panel B. Cresyl violet (Nissl) staining did not indicate any neurotoxic damage in the area surrounding the probe tip following the perfusion of ethanol (Panel C). The damage was only observed in a restricted region immediately proximate to the tip of the probe.

Adenosine Measurements

Panel A, B and C in Figure 2 illustrate adenosine chromatograms separated by the HPLC system and detected by the UV detector. Figure 2 Panel A describes the peak of pure adenosine (standard). 10 μ L of 50 nmol/L of standard adenosine was injected into the HPLC resulting in a well resolved and easily detectable adenosine peak with signal to noise ratio greater than 2:1. This result suggests that our assay was very sensitive with a detection limit of 500 fmol. Panel B and Panel C in Figure 2 describes adenosine in 10 μ L samples collected from the BF during aCSF and 100 mM ethanol perfusion respectively. Adenosine peaks were well resolved and easily detectable suggesting that adenosine levels in the BF were detectable and measurable by our system and ethanol perfusion did not affect or interfere with the retention time of adenosine peaks. In addition, the chromatogram in Figure 2 Panel C demonstrates increased adenosine peak during 100 mM ethanol perfusion as compared to adenosine peak during aCSF perfusion.

Effect of local ethanol perfusion on adenosine release

The mean ($\pm$ SEM) concentration of adenosine in 10 μ L microdialysate collected from the BF during baseline conditions (aCSF perfusion) was 260 ± 91 fmol (or 26.9 nmol/L). This value is comparable to the previously reported adenosine level in the microdialysate sample collected from the BF (see Porkka-Heiskanen et al., 1997). As described in Figure 3, local microdialysis perfusion of ethanol in the BF produced a significant increase in extracellular adenosine ($F=3.0$; df (total) = 39; $p<0.05$; one way repeated measures ANOVA). Subsequent post-hoc analysis (Bonferroni's test) revealed that perfusion of the lowest dose of 30 mM ethanol into the BF produced a trend level increase (non-significant) in extracellular adenosine with the mean ($\pm$ SEM) adenosine levels rising to $521 (\pm 175)$ fmol/10 μ L. However, perfusion of 100 mM and 300 mM doses of ethanol into the BF produced significant ($p<0.05$) increases in extracellular adenosine levels as compared to during pre-ethanol aCSF perfusion. The 100 mM dose of ethanol increased the mean ($\pm$ SEM) extracellular adenosine levels to $973 (\pm 408)$ fmol/10 μ L. This reflects an increase of approximately 375% from baseline (pre-ethanol aCSF) value. Although, the 300 mM dose of ethanol perfusion in the BF further increased extracellular adenosine to achieve a mean ($\pm$ SEM) value of 1013 ± 480 fmol/10 μ L, a rise of approximately 400%, the increase appeared to plateau out. Finally, ethanol induced increased extracellular adenosine levels in the BF started to decline during post-ethanol aCSF perfusion (Mean $\pm$ SEM = 573 ± 256 fmol/10 μ L).

In vitro recovery

The mean $\pm$ SEM *in vitro* recovery of microdialysis probes was $11.3 \pm 1.7\%$. Based on our *in vitro* recovery data, the estimated ethanol concentration around the vicinity of the probe tip in the BF would be lowered to approximately 11% of perfusion concentration. Thus, perfusion of 30, 100 and 300 mM of ethanol would result in an effective ethanol concentration of approximately 3.3, 11.0 and 33 mM in the BF around the vicinity of the probe.

DISCUSSION

This is the first study to demonstrate that ethanol acts directly in the brain to increase extracellular levels of adenosine. In this study, we examined the effects of reverse microdialysis perfusion of ethanol on extracellular levels of adenosine in the BF. The results of our study suggest that 1) local reverse microdialysis administration of ethanol into the BF produce a significant increase in extracellular levels of adenosine in the BF. 2) The effects of ethanol were not confounded by a general toxicity, as measured by cresyl violet and the continued presence of ChAT staining.

Microdialysis perfusion of three doses of ethanol entails syringe changes coupled with frequent sample collections that can disturb the animal and cause partial sleep deprivation. Sleep deprivation is known to affect adenosine levels in the BF (reviewed in Basheer *et al.* 2004). In order to avoid this confound, we conducted our experiment during the first 5 hr after dark onset. The rats are maximally awake during this period and therefore unlikely to be sleep deprived.

The use of a microdialysis probe for reverse microdialysis to locally apply drugs in specific regions of the brain provides precise control over the concentration and duration of drug administration. It offers several advantages over other techniques including the ability to deliver very low and constant concentrations of drugs, thus reducing the probability of neurotoxic damage (Portas *et al.* 1996; Quan and Blatteis 1989; Thakkar *et al.* 2003b; Thakkar *et al.* 1998). This technique has been effectively used previously to deliver ethanol locally into selected brain region and measure neurotransmitter release without causing any neurotoxic damage (Ericson *et al.* 2003; Lof *et al.* 2007).

We used the HPLC-UV detection system for adenosine measurements from microdialysate sample. Our method has been verified by us and others, and has been described in number of published reports (Porkka-Heiskanen et al., 1997; McKenna et al., 2007; Kalinchuk et al., 2008; Murillo-Rodriguez et al., 2004; Blanco-Centurion et al., 2006; Murillo-Rodriguez et al., 2008). In addition, the use of retention time, area under the peak, and absorbance wavelength for identification and quantification of a compound are standard methods used in HPLC-UV (see Hanai, 1999). A detailed review of other factors that affect microdialysis sampling has been published previously (Watson et al. 2006).

Although microdialysis perfusion of ethanol produced a concentration dependent increase in extracellular levels of adenosine in the BF, we did not perform a detailed pharmacological dose response study. In this initial report, our objective was to evaluate pharmacologically relevant doses of ethanol on adenosine release, *in vivo*, in intact animals. Pharmacological relevancy of ethanol doses was estimated based on *in vitro* recovery studies. Based on our probe recovery studies with adenosine, we estimated that the concentrations of ethanol administered with reverse microdialysis will be lowered to approximately 11% in the BF surrounding the probe. Thus, perfusion of ethanol 300 mM, our highest dose, will result in a concentration of 33 mM in the BF directly outside the probe. This concentration of ethanol is in the range of ethanol concentration observed in the brain after a systemic administration of 2.5 g/kg ethanol (Ericson et al. 2003; Yoshimoto and Komura 1993). Thus, ethanol doses used in this report are pharmacologically relevant. As described in the introduction, there is consistent evidence demonstrating the role of adenosine in mediating many neuronal responses to ethanol, however, the source of adenosine is yet unknown. The results of our study demonstrates, for the first time, that ethanol acts directly in the brain to increase extracellular adenosine, most likely by inhibiting the adenosine transporter ENT1 as demonstrated by Nagy and her coworker in cell cultures (Nagy et al., 1990). Furthermore, our data also suggest that ethanol perfusion in the BF had profound effect on extracellular levels of adenosine. The highest dose of 300 mM (effective concentration in the BF near the probe = 33 mM) produced approximately 4 fold increase in adenosine. This may suggest a strong and a unidirectional (influx) inhibition of adenosine transporter by ethanol (Dunwiddie 1999).

The results of this study suggest that while 100 and 300 mM doses of ethanol perfusion in the BF increased extracellular adenosine, the increase appeared to plateau out during 300 mM ethanol perfusion. This may be due to adaptation caused by more than 3 hr of continuous ethanol perfusion.

Does ethanol increase adenosine levels in other brain regions? This is an important question left unanswered. We believe that ethanol will increase adenosine levels in other brain regions. For example ethanol induced increased adenosine in the cerebellum may be responsible for motor impairments (Meng and Dar 1995; Dar and Mustafa 2002; Dar 2002; Dar 1997).

Finally, ethanol induced increased extracellular adenosine in the BF reveals a putative mechanism for ethanol induced increase in NREM sleep. It is well established that acute ethanol intake promotes sleep in humans and animals (Roehrs and Roth 2001; Brower 2001; Kubota et al. 2002). However, the cellular mechanisms responsible for the somnogenic effects of ethanol are yet unknown. Consistent evidence exist to suggest that adenosine acts via A₁ receptor to inhibit the wake-promoting neuron in the BF resulting in the promotion of sleep (Porkka-Heiskanen et al. 1997; Alam et al. 1999; Thakkar et al. 2003b; Thakkar et al. 2003a; Blanco-Centurion et al. 2006). Therefore, ethanol induced increased adenosine in the BF, as described in this report, may be responsible for sleep promoting effects of ethanol. However, further work is necessary to verify this role.

In conclusion, we have shown that local reverse microdialysis administration of ethanol in the BF produced a profound increase in extracellular adenosine. This is the first study to demonstrate that ethanol acts directly in the brain to increase adenosine release.

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Figure 1. Local ethanol perfusion increased extracellular adenosine in the BF

Panel A describes a schematic representation of the anatomical location of histologically identified microdialysis probe tip sites. All sites (black circles) were located between antero-posterior -0.3 and -0.6 and are mapped onto one side of the coronal brain section (-0.4 , adapted from Paxinos and Watson, 2007). The side (left or right BF) of probe implantation was counterbalanced to avoid any side (left vs right) effects. In addition, the same unilateral microdialysis probe was used for ethanol perfusion and adenosine sample collection in the cholinergic BF. Abbreviations: ac = anterior commissure; HDB = Horizontal diagonal band; MCPO = Magnocellularis preoptic; SI = Substantia inominata. For identification of unlabeled structures see Paxinos and Watson (2007).

Panel B. A representative photomicrograph depicting the lesion caused due to implantation of microdialysis probe in the BF (large arrowhead). The tip of the probe is surrounded by ChAT +ve cholinergic neurons in the BF (small arrows). Calibration bar = $100\ \mu\text{m}$.

Panel C. Cresyl violet staining revealed that the damage is constrained to the immediate vicinity of the microdialysis probe track (black arrow) and intact neurons are clearly visible surrounding the tract. Calibration bar = $100\ \mu\text{m}$

Figure 2. Representative chromatograms from adenosine assay

Panel A describes standard adenosine chromatogram. 10 μ L of pure adenosine sample (50 nmol/L) was injected into the HPLC and detected by the UV detector (wavelength = 258 nm). The adenosine peak was well resolved and easily detectable (signal to noise ratio > 2:1) suggesting that the detection limit of our adenosine assay was 500 fmol.

Panel B describes adenosine peak (690 fmol; Animal #186) from a 10 μ L microdialysate sample collected from the BF during aCSF perfusion.

Panel C describes adenosine peak (1453 fmol; Animal #186) from 10 μ L microdialysate sample collected from the BF during 100mM ethanol perfusion.

Figure 3.

The effect of reverse microdialysis administration of ethanol (three doses) into the BF on extracellular adenosine. One way repeated measures ANOVA revealed that microdialysis delivery of ethanol to the BF significantly increased extracellular adenosine. *Post-hoc* Bonferroni's statistic showed that 100 and 300 mM doses of ethanol produced a significant (* = $p < 0.05$) increase in extracellular adenosine in the basal forebrain.