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Effects of Ethanol and Pentobarbital on Neuronal Hexose Uptake in Inbred Mice

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MORROW, E. L. AND M. A. MEDINA. *Effects of ethanol and pentobarbital on neuronal hexose uptake in inbred mice.* PHARMACOL BIOCHEM BEHAV **24**(1) 55–60, 1986.—The effect of ethanol and pentobarbital narcosis on 2-deoxyglucose uptake into brain synaptosomes prepared from inbred C57BL/6J and DBA/2J mice which exhibit differential central sensitivity to ethanol and heterogenous ICR mice was examined. A reversible depression of synaptosomal uptake was exhibited in all strains administered ethanol acutely, occurring at 2 min in ICR and C57BL/6J mice and 15 min in DBA/2J. Uptake returned to control values in all strains at 30 min although the mice remained intoxicated. Brain glucose concentration was significantly elevated at this time. Pentobarbital administration was without effect on synaptosomal hexose transport in DBA/2J and C57BL/6J mice but increased it significantly in ICR mice at 30 min. Pentobarbital anesthesia did not alter brain glucose concentration. No correlation was apparent between synaptosomal 2-deoxyglucose uptake and differential CNS sensitivity to ethanol and pentobarbital. The effects of ethanol and pentobarbital on neuronal hexose transport is discussed with respect to reported changes in glycolytic metabolism produced by these agents.

2-Deoxyglucose Synaptosomes Neuronal uptake Ethanol Pentobarbital ICR C57BL/6J DBA/2J Glucose

THE effect of ethanol on the metabolism of glucose in the central nervous system of mice and rats has been examined by many investigators. In a variety of central neuronal preparations treated either *in vivo* or *in vitro* with ethanol, glucose consumption decreased as did lactate formation [8, 20, 30, 36]. Brain concentrations of glucose and glucose-6 phosphate increased following acute administration of intoxicating doses of ethanol in fed and fasted rat [20,33] and fed mice [24]. The effect of ethanol to increase brain glucose and glucose-6-phosphate appears to be correlated with the measured decrease in glucose utilization.

Similar effects on central glycolytic metabolism are seen in barbiturate anesthetized mice and rats. *In vivo* glucose utilization was decreased by 55% in mice and rats anesthetized with phenobarbital [10,14]. The uptake of 2-deoxyglucose (2-DG) increased in brain slices obtained from rats 4 hours following phenobarbital administration [22]. Corresponding to the decreased *in vivo* glucose utilization, brain concentrations of glucose and glucose-6-phosphate increased in barbiturate anesthetized animals [15,18].

Although the disruption in cerebral glycolytic metabolism demonstrated during ethanol intoxication and barbiturate anesthesia is well documented, no specific locus responsible for the production of this effect has been defined. A variety of sites of interruption are suggested by the observed changes in glycolytic metabolites. Increased brain concentration of glucose suggests that uptake into brain from blood and into the neuron may be involved. Alternatively, increased brain glucose concentration could be the result of decreased glucose utilization following entry of glucose into the nerve cell.

Transport of the structural analogue of glucose 2-deoxy-D-glucose (2-DG), across synaptic membranes has been demonstrated in rat and guinea pig brain synaptosomes [6,11]. Transport into nerve endings is characterized as a high affinity carrier mediated, stereospecific and saturable process [6]. Transport is considered an important site of metabolic regulation of glucose.

The purpose of this investigation was to examine the influence of ethanol and pentobarbital on synaptosomal uptake of 2-DG to determine if alteration in this parameter is the means by which brain glucose concentration is increased during narcosis by these agents. It is generally believed that ethanol exerts its effect in the central nervous system by altering membrane fluidity [25], an action which is hypothesized to alter membrane protein function [31]. Since carrier-mediated delivery of glucose to the nerve ending is a membrane-associated site of regulation of glucose utilization, it seems a possible site at which ethanol might alter cerebral glucose utilization. Furthermore, since the adult mammalian brain is almost totally dependent on glucose as its source of energy, also examined was the possibility

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that alteration in central glucose utilization at the site of its cellular uptake may be in part responsible for the narcosis produced by these two centrally depressant agents. To this end, inbred mouse strains DBA/2J and C57BL/6J which differ in central sensitivity to ethanol and pentobarbital [5, 13, 23, 28] as well as the heterogenous ICR strain were utilized in this study.

METHOD

Male ICR, C57BL/6J and DBA/2J mice weighing 18–22 g were obtained from ARS/Sprague Dawley (Madison, WI). Due to unavailability of DBA/2J mice in the breeding facility, an additional group of male DBA/2J mice weighing 18–22 g from an identical stock were obtained from Jackson Laboratories (Bar Harbor, ME) for the pentobarbital studies. Mice were allowed food and water ad lib except for those used in analysis of blood and brain drug and glucose concentrations. These animals were fasted 24 hr prior to drug administration.

Drug Administration

Animals were injected intraperitoneally with 4 g/kg ethanol (National Distillers and Chemical Corp., New York, NY) as a 34.5% (v/v) solution in Tris-saline pH 7.4 or 50 mg/kg sodium pentobarbital (Abbott Laboratories, Chicago, IL) in saline. Control animals received an equivalent volume of the appropriate injection vehicle.

Sleep Time Studies

Behavioral sensitivity to ethanol and pentobarbital was assessed by measuring pharmacological sleep time following drug administration. Sleep time was measured from the time animals lost their righting reflex to the time at which this response was regained. Mice were considered to have regained their righting reflex when they could right themselves three times in 30 sec.

Analysis of Blood and Brain Glucose, Ethanol and Pentobarbital Concentration

Ethanol, pentobarbital or the appropriate vehicle was administered as described. Animals were sacrificed by microwave irradiation (300 msec, 5.5 kW) 30 min following drug administration [14]. Immediately prior to sacrifice, blood samples were collected by retro-orbital sinus puncture. All blood was collected in heparinized tubes. Brain and blood samples were stored at −20°C until assayed.

Ethanol levels in blood were measured by gas chromatography using a head space technique [34]. Pentobarbital levels in blood and brain were determined by capillary gas-liquid chromatography [35]. Glucose concentration in brain was measured by a spectrophotometric enzyme coupling method [16]. Protein concentration was estimated with a Technicon Autoanalyzer (Technicon Instruments, Carrollton, TX) using the folin phenol reagent [17].

Synaptosomal Uptake of 2-Deoxyglucose

Control and drug-treated mice were sacrificed by cervical dislocation at 2, 15 or 30 min following ethanol administration or 15 or 30 min following pentobarbital administration. All treated animals had lost their righting reflex at the time of sacrifice.

Synaptosomes were prepared using differential and density gradient centrifugation [2] with minor modifications of the standard protocol as follows. All centrifugations were conducted at 0°C and solutions were ice cold. Superficial blood vessels were carefully removed from the brain surface. Three brains were pooled to comprise one sample and hand homogenized in 9 volumes of 0.32 M sucrose. The homogenate was centrifuged for 10 min at 1000×g.The supernatant obtained was reserved and the pellet washed and recentrifuged for 10 min at 1000×g. The resulting supernatant was combined with that reserved and centrifuged for 20 min at 17,000×g. The pellet obtained was washed and recentrifuged for 20 min at 17,000×g. The crude mitochondrial fraction (P_2) resulting was resuspended in 5 ml 0.32 M sucrose. This suspension was applied to a discontinuous Ficoll-sucrose gradient consisting of 13 ml layers of 12% (w/v) and 7.5% (w/v) Ficoll (Sigma Chemical Co., St. Louis, MO) in 0.32 M sucrose. Centrifugation was carried out in an L2-65B ultracentrifuge using an SW-27 rotor (Beckman Instrument, Inc., Palo Alto, CA) at 60,000×g for 90 min. The synaptosomal fraction was obtained at the 7.5%–12% Ficoll-sucrose interface. This fraction was diluted with one volume of 0.32 M sucrose and pelleted at 15,000×g for 20 min. The final synaptosomal pellet was resuspended in 1 ml 0.32 M sucrose and used immediately in uptake studies.

Uptake of 2-DG was determined by radiofiltration assay [6]. Aliquots of the synaptosomal suspension (0.1 ml) containing approximately 0.25 mg protein were incubated for 15 min at 30°C in 0.5 ml of buffer composed of 265 mM sucrose and 26 mM potassium phosphate pH 7.4. The reaction was initiated by the addition of 0.05 ml 4 mM 2-DG (Sigma Chemical Co., St. Louis, MO) containing ^{3}H-2-DG (New England Nuclear, Boston, MA) (0.1–0.2 mCi/nmole). Non-specific entry of 2-DG into synaptosomes was measured under identical conditions with 200 mM glucose replacing an equivalent concentration of sucrose in the assay buffer. Uptake was terminated at 15 min with the addition of 5 ml ice-cold 0.32 M sucrose and immediate filtration of samples over 0.45 μm filters (Amicon Corp., Danvers, MA). The filters were washed four times with 5 ml ice-cold 0.32 sucrose, air dried, placed overnight in 20 ml ACS scintillant (Amersham Corp., Arlington Heights, IL) and counted the following morning by liquid scintillation spectrometry.

Statistics

Student's unpaired (two-tailed) t-test was used in the evalu-

TABLE 1

EFFECT OF ETHANOL AND PENTOBARBITAL ON Rrighting REFLEX IN ICR, C57BL/6J AND DBA/2J MICE

Strain	Sleep Time (Min)	
	Ethanol	Pentobarbital
ICR	119 ± 27 (9)	49 ± 17 (5)
C57BL/6J	68 ± 16 (10)*	45 ± 10 (14)
DBA/2J	109 ± 22 (12)	56 ± 13 (14)

Each animal was injected IP with ethanol (4 g/kg) or pentobarbital (50 mg/kg).

Sleep time was measured from the time animals lost their righting reflex until the time it was regained.

Values represent mean ± S.E. Number of animals utilized is indicated in parentheses.

*Significantly different from ICR and DBA/2J at $p \leq 0.01$.

TABLE 2

BRAIN GLUCOSE CONCENTRATION FOLLOWING ETHANOL OR PENTOBARBITAL ADMINISTRATION

Strain	μmoles/g brain Control	Ethanol	Pentobarbital
ICR	1.55 ± 0.29 (5)	3.12 ± 0.63 (5)*	2.07 ± 0.48 (5)
C57BL/6J	1.88 ± 0.31 (5)	3.83 ± 0.89 (4)†	1.89 ± 0.80 (4)
DBA/2J	1.04 ± 0.13 (5)	1.47 ± 0.30 (5)‡	1.25 ± 0.14 (4)

Animals were fasted 24 hours prior to drug administration. At this time they received ethanol (4 g/kg), pentobarbital (50 mg/kg) or an equivalent volume of the injection solution. Animals were sacrificed 30 minutes following drug administration.

Values are mean ± S.E. The number of animals utilized is indicated in parentheses.

*Significantly different from control at $p \leq 0.001$.

†Significantly different from control at $p \leq 0.005$.

‡Significantly different from control at $p \leq 0.02$.

TABLE 3

EFFECT OF *IN VIVO* ETHANOL TREATMENT ON 2-DEOXY-D-GLUCOSE UPTAKE IN BRAIN SYNAPTOSOMES OF ICR, C57BL/6J AND DBA/2J MICE

Strain	Synaptosomal Uptake (nmoles/mg/min) Control	2 min	15 min	30 min
ICR	0.269 ± 0.043 (21)	0.199 ± 0.053 (9)*	0.280 ± 0.026 (10)	0.311 ± 0.039 (14)
C57BL/6J	0.323 ± 0.038 (20)	0.238 ± 0.062 (7)*	0.315 ± 0.043 (11)	0.324 ± 0.032 (11)
DBA/2J	0.337 ± 0.154 (20)	0.317 ± 0.068 (7)	0.232 ± 0.054 (11)†	0.270 ± 0.020 (10)

Animals injected with ethanol (4 g/kg) and sacrificed at indicated time intervals.

Values are mean ± S.E. The number in parentheses indicates the number of experiments per group.

*Significantly different from control at $p \leq 0.015$.

†Significantly different from control at $p \leq 0.004$.

ation of sleep-time, glucose concentration and drug levels. Uptake studies were evaluated with a 2-way analysis of variance and statistical significance determined with a Student-Newman-Keul multiple comparison test.

RESULTS

Sleep Time

Table 1 illustrates sleep time scores obtained in ICR, C57BL/6J and DBA/2J mice under ethanol or pentobarbital narcosis. All ethanol treated animals lost their righting reflex 2 min following drug administration. ICR and DBA/2J mice slept significantly longer than C57BL/6J mice at the dose of ethanol tested. No difference in behavorial sensitivity between ICR and DBA/2J mice was manifested with ethanol narcosis. At the dose of pentobarbital tested, all strains slept an equivalent period of time.

Blood and Brain Glucose, Ethanol and Pentobarbital Concentration

No significant differences in blood ethanol levels were observed among mouse strains. The mean blood ethanol level was 343±66 mg% 30 min postinjection indicating a significant level of intoxication in these animals.

Pentobarbital concentration 30 min postinjection in blood of DBA/2J mice (18.4±2.5 μg/ml) was significantly higher than that measured in ICR (11.5±5.6 μg/ml) or C57BL/6J (12.5±4.4 μg/ml) mice. Brain pentobarbital levels were also higher in DBA/2J (41.5±5.8 μg/g) than in ICR (21.5±6.2 μg/g) or C57BL/6J (19.9±6.9 μg/g). No difference in blood or brain drug concentration was seen between ICR and C57BL/6J mice.

Brain glucose concentration increased in all mouse strains 30 min following ethanol administraton (Table 2). However, no change in brain glucose concentration was seen in any mouse strain following 30 min of barbiturate anesthesia.

Synaptosome Uptake of 2-Deoxyglucose

The effect of acute ethanol intoxication on high affinity synaptosomal 2-DG uptake was examined at 2, 15 and 30 min following drug administration to allow comparison of alteration of substrate transport to the time course of behavioral manifestations of ethanol intoxication. As illustrated in Table 3, ethanol significantly inhibited synaptosomal uptake of 2-DG in ICR and C57BL/6J mice 2 min following drug administration. In DBA/2J mice uptake was depressed only after 15 min. Uptake had returned to control values at 15 min in ICR and C57BL/6J mice. At 30 min, all strains exhibited a recovery of uptake. Although not shown here, no change was noted in the diffusional component of 2-DG entry into synap-

TABLE 4
EFFECT OF *IN VIVO* PENTOBARBITAL TREATMENT ON 2-DEOXY-D-GLUCOSE UPTAKE IN BRAIN SYNAPTOSOMES OF ICR, C57BL/6J AND DBA/2J MICE

Strain	Synaptosomal Uptake (nmoles/mg/min)		
	Control	15 min	30 min
ICR	0.286 ± 0.047 (9)	0.238 ± 0.055 (7)	0.387 ± 0.068 (9)*
C57BL/6J	0.261 ± 0.077 (15)	0.247 ± 0.052 (15)	0.280 ± 0.053 (14)
DBA/2J	0.365 ± 0.054 (12)	0.337 ± 0.036 (12)	0.352 ± 0.052 (12)

Animals were injected with pentobarbital (50 mg/kg) and sacrificed at the indicated time intervals.

Values are mean ± S.E. The number in parentheses indicates the number of experiments per group.

*Significantly different from control at $p \leq 0.004$.

tosomes. It remained less than 5% of specific uptake under these conditions.

As with ethanol, the effect of pentobarbital (50 mg/kg) anesthesia on synaptosomal 2-DG uptake was examined at varying time intervals following drug administration. No effect on synaptosomal uptake of 2-DG was seen at 15 or 30 min following pentobarbital administration in C57BL/6J or DBA/2J mice or in ICR mice at 15 min (Table 4). However, pentobarbital narcosis resulted in a significant increase in 2-DG uptake in synaptosomes isolated from ICR mice at 30 min compared to control.

DISCUSSION

The differential behavioral response to acute ethanol intoxication in the inbred mouse strains studied here is comparable to reports of other investigators [5,13]. It is attributed to differential neuronal sensitivity of these strains and not to variation in rate of ethanol metabolism [26,27]. The absence of differences in blood alcohol levels measured in this investigation concurs with this conclusion.

In regard to pentobarbital-induced narcosis, the equivalent sleep time scores observed in these mouse strains agrees with reports of other investigators [23,28]. The significantly higher blood and brain pentobarbital levels measured in DBA/2J mice as compared to the C57BL/6J strain is also in agreement [23,28]. These results indicate a centrally mediated differential sensitivity to pentobarbital since sleep time scores were comparable in the presence of significantly different blood and brain drug concentrations. The CNS sensitivity to pentobarbital in DBA/2J and C57BL/6J mice is opposite to that observed with ethanol. The difference in response to ethanol and pentobarbital between these strains may be attributed to different central sites of action of these drugs [23]. However, it may also be due to differences in response of neural function at a defined locus to the action of these drugs. Therefore, these animals seemed an appropriate model with which to examine the behavioral and biochemical consequences of ethanol and pentobarbital narcosis on the uptake of glucose into the nerve cell.

Carried mediated transport of 2-DG has been well described in rat brain synaptosomes [6]. Uptake of 2-DG in mouse brain synaptosomes exhibits characteristics similar to those of rat including high-affinity, specificity, saturability and a dependence on temperature, time and synaptosomal protein concentration. Under the defined conditions of the assay, *in vivo* ethanol administration produced a time dependent and reversible depression of specific 2-DG uptake. This effect of ethanol appears not to be correlated with the behavioral manifestation of intoxication, loss of righting reflex, in ICR, C57BL/6J or DBA/2J mice. All animals had lost their righting response 2 min following ethanol administration. Although uptake was depressed in ICR and C57BL/6J mice at this time, DBA/2J mice lost their righting response without any change in transport activity.

It also appears that no correlation exists between 2-DG uptake and differential neuronal sensitivity to ethanol intoxication in these mouse strains. Recovery of hexose uptake had occurred in all strains at 30 min following drug administration, although the animals were still narcotized. Since differential neuronal sensitivity is not manifested until recovery of the righting response [13], it is unlikely that these factors are related. Differences in brain sensitivity of heterogenous mice selectively bred for sensitivity or resistance to the acute actions of ethanol is also not due to differential alteration in the rate of cerebral glucose utilization by ethanol [30].

The early and transient effect of ethanol on glucose uptake also appears to be unrelated to the ethanol-induced increase in brain glucose concentration demonstrated in all mouse strains in this investigation. Brain glucose concentration is reported to increase as early as 5 min following drug administration [24] and to remain elevated up to 13 hours [33]. Increased brain glucose levels during anesthesia are reported to be intracellular [21]. Ethanol's effect to reversibly depress synaptosomal hexose uptake early in the time course of intoxication would serve to decrease glucose concentration intracellularly. However, the measured decrease in rate of cerebral glucose utilization [20,30] may be of greater consequence in elevating cerebral glucose concentration during ethanol-induced narcosis than the early transient change in intracellular substrate transport.

In contrast to ethanol, an effect of pentobarbital anesthesia on synaptosomal hexose uptake was demonstrated only in ICR mice, only at 30 min and was opposite in effect to that produced by ethanol. This difference in effect between ethanol and pentobarbital narcosis on synaptosomal uptake of 2-DG is not readily explainable nor is the selective effect on synaptosomes isolated from ICR mice. In this investigation no change in brain glucose concentration occurred during pentobarbital anesthesia confirming the previous observation that alterations do not occur at cerebral pentobarbital levels less than 45 μg/g [3]. Significant elevations are produced with barbiturate doses much higher than those used in our studies [15,18].

It has been demonstrated that brain glucose utilization is decreased during ethanol intoxication or barbiturate anesthesia [3, 14, 20]. However, both drugs induce hypothermia which itself reduces glucose utilization [19]. Thus any correlation between *in vitro* synaptosomal glucose uptake and the *in vivo* CNS sensitivity of the mouse strains tested may be obscured by this hypothermic component. This may not be a problem with pentobarbital since the dose used (50 mg/kg) lowers glucose utilization by 50% and causes loss of righting reflex, but does not result in a significant decrease in body temperature [3]. However the concentration of ethanol given in our experiments will lower body temperature by 2–3°C after 30–60 min, therefore one cannot rule out the possibility that hypothermia may be a factor in this case [7,29].

Increased membrane fluidity which has been demonstrated following *in vitro* exposure of mouse synaptosomal membranes [1] and phospholipid vesicles [32] to pharmacologically relevant concentrations of ethanol does not appear to be the mechanism responsible for the alteration in synaptosomal uptake of 2-DG in this investigation. If this were so, it would be expected that the diffusional component of 2-DG entry into synpatosomes would be modified as well as the carrier mediated component since the activity of both is defined by membrane integrity. However, no change was noted in the diffusional component of transport. Assuming narcosis is produced as a consequence of increased membrane fluidity, although a recent investigation gives evidence to the contrary [12], it would be expected that the time course of narcosis would be comparable to the time course of alteration of synaptosomal hexose uptake. This was not the case, ethanol-induced depression of 2-DG uptake was reversed prior to the animals' recovery of righting response. However, it is possible that ethanol-induced alteration of 2-DG transport into synaptosomes is occuring at locations other than those involved in righting response which might account for the lack of similarity in time course of these two events.

Investigations have demonstrated that glucose influx into various brain regions is correlated with regional glucose utilization, the rate of which varies throughout brain structures [4,9]. Since this investigation examined hexose uptake only in whole brain preparations, it is conceivable that alteration of this parameter within discrete brain regions might be masked. Further investigation is required to clarify this.

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