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Acute Ethanol Effects on Focal Cerebral Ischemia in Nonfasted Rats

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Focal cerebral ischemia was induced in a rat model of middle cerebral artery occlusion. Three groups of adult male Sprague-Dawley rats, given food and water ad libitum, were subjected to 4 hr of middle cerebral artery occlusion. All were given vehicle control and ethanol pretreatments intraperitoneally 1 hr before. Mean ipsilateral brain water content in the control, 2 g/kg ethanol, and 2 g/kg ethanol + insulin-treated groups showed: ischemia core: 81.1%, 82.5%, and 80.9%; intermediate zone: 81.0%, 81.9%, and 80.3%; and outer zone: 80.3%, 81.3%, and 80.1%, respectively. Brain Na^+ and K^+ content in these groups paralleled the water content. In addition to significantly ($p < 0.05$) more brain edema, the 2 g/kg ethanol-treated animal group also had significant hyperglycemia. In contrast, the 2 g/kg ethanol + insulin-treated animals were normoglycemic and had ischemic, intermediate, and outer zone Na^+, K^+, and Cl^- levels comparable with the control group ($p > 0.05$). These results stress the importance of measuring and controlling plasma glucose levels in the in vivo studies of the neurotoxic effects of acute ethanol.

Key Words: Ethanol, Hyperglycemia, Insulin, Ischemia.

IN VIEW of the widespread misuse of ethanol, it is surprising so little is known about its effects on acute brain ischemia. There is also a lack of data on whether the hyperglycemia that results when ethanol is administered to nonfasted animals contributes to acute brain injury. The goal of the present research was to determine the effects acute ethanol intoxication on focal cerebral ischemia in nonfasting rats in a highly reproducible model of middle cerebral artery occlusion (MCAO).[1–3] The experiments were designed to mimic a human scenario of two ethanol intoxicated males and one male teetotaler (i.e., a person who does not drink ethanol). All three had meals before an identical MCAO. The question to be answered was which had more or less brain edema when examined 4 hr later. The hypothesis tested is that ethanol-induced hyperglycemia in vivo contributes, in part, to its acute neurotoxicity. Brain edema is a major complication and cause of death after a stroke. The development of ischemic brain edema is related to an increase of water and Na^+ and a decrease of K^+. Edema formation was chosen because this is a major indicator of quality of survival in patients with brain ischemia.

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MATERIALS AND METHODS

Experimental Protocol

This study was approved by the University of Michigan Committee on the Use and Care of Animals. Twenty-one adult male Sprague-Dawley rats weighing 230 to 300 g (Charles River, Portage, MI) were randomly divided into three groups to undergo MCAO for 4 hr. They were on a 0700 to 1900 light and 1900 to 0700 dark cycle on a standard rodent diet and water ad libitum. They were pretreated 60 min before MCAO as follows: group 1 were controls who received 5% glucose solution 10 mg/kg ip; group 2 received 20% ethanol in 5% glucose solution in a dose of 2 g/kg ip; and group 3 received 20% ethanol in a dose of 2 g/kg ip +insulin. Small doses of insulin (IU) were given intravenously to maintain normoglycemia during MCAO. During all experiments, physiological parameters were monitored and maintained in the normal range. Local cerebral blood flow (CBF) was measured through the surface of the cortex using a laser Doppler flowmeter monitor (Vasamedics, Inc., St. Paul, MN) equipped with a small caliber probe of 0.7 mm in diameter (P-433, Vasamedics). Rectal temperature was measured by a thermometer (YSI, model 73A; Yellow Springs Instrument Co., Yellow Springs, OH). The temperature was carefully regulated by heating lamp and heating pad to maintain 37°C. After 4 hr of MCAO, each animal was killed and brain samples measured for water and ion content. The 100 units/ml of insulin was diluted with 0.9% NaCl to a 1 unit/ml solution.

Rat MCAO Suture Model

Anesthesia was induced by inhalation with 5% isoflurane in 0.7 liters/min: O_2/air gas mixture. After tracheal intubation, the lungs were mechanically ventilated to maintain the PaO_2 90 mm Hg or above. Anesthesia was continued with 1.5% isoflurane. The femoral artery was cannulated with PE-50 tubing to allow continuous monitoring of arterial blood pressure and sampling of arterial gases, blood pH, and blood glucose. Arterial blood pressure was maintained above 90 mm Hg by adjusting the isoflurane concentration. MCAO was produced as described previously.[1–3] Briefly, under an operating microscope, the left common carotid artery was exposed through a midline incision. The branches of the external carotid artery (ECA) were isolated and coagulated along with the terminal lingual and maxillary artery branches. The internal carotid artery (ICA) was then isolated and its extracranial branch, the pterygopalatine artery, was ligated close to its origin. The ICA remained patent. A 3-cm length of 3-0 nylon suture with a slightly enlarged and rounded tip was introduced into the transected lumen of the ECA and gently advanced from the ECA into the ICA. The distance from the tip of the suture to the bifurcation of the common carotid artery was 19 to 20 mm in these rats.

CBF Measurement

Laser Doppler flow was determined at two cortical sites. Point A was placed 6 mm lateral in the contralateral hemisphere, and point B was placed 6 mm lateral in the ipsilateral ischemic hemisphere. Both sites were 1.5 mm posterior to the bregma. After mounting the rat in a stereotaxic frame, the skull was exposed through a midline skin incision. Two 2.0 mm holes were drilled such that a thin bone layer could be carefully removed to prevent injury to the cortex. The dura maintained intact. The probe was held in a micromanipulator and stereotactically advanced to gently touch

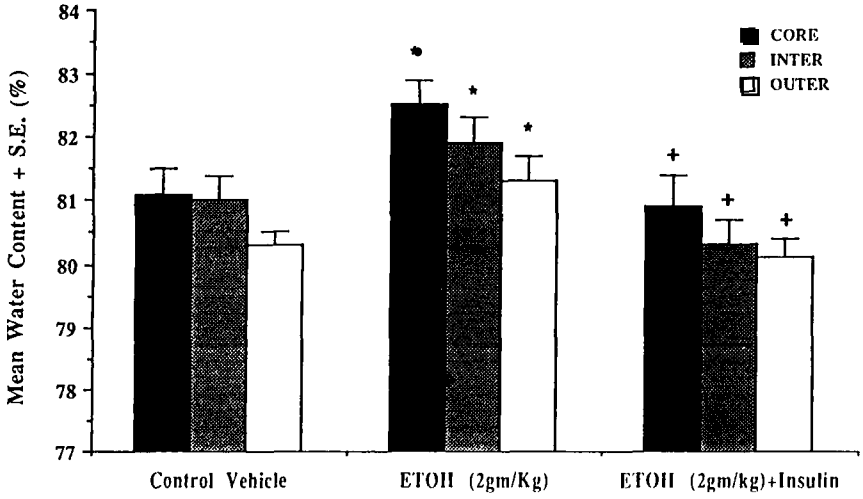

Fig. 1. Effect of ethanol (ETOH) on mean water content of ischemic brain tissue after MCAO. The mean water content + SE is plotted for the ipsilateral brain for the ischemic core and inner and outer penumbra zones. The groups to the left are control vehicle rats made ischemic. A dose of 2 g/kg ip of ethanol 1 hr before produced a statistically significant increase (*$p < 0.05$) in brain edema that was reversed to control ischemic levels ($p < 0.05$) in the third group of rats given 2 g/kg ip of ethanol 1 hr before + 0.25 to 0.5 units/kg of insulin to maintain normoglycemia. The latter group was statistically significantly different (+$p < 0.05$) from the ethanol alone-treated group. In this and subsequent figures, all rats were nonfasted before 4 hr MCAO.

the intact dura mater. To obtain a clear optical medium between the flow probe and the cortex, and to maintain the brain temperature at 37° to 37.5°C, warmed 0.9% NaCl was slowly perfused around the probe during the experiment. Ten minutes of stable baseline flow readings were obtained before occlusion. Then, MCAO was produced and the digital display of CBF recorded. The CBF values were calculated and expressed as percentage of baseline values (milliliters per 100 grams per minute). If CBF was not maintained below 35 ml/100 g/min during occlusion, the animal was excluded from the study.

Water, Na^+, K^+, and Cl^- Content

Samples were removed using 7 and 10 mm cork borers from the core, intermediate, and outer zones of the ischemic cerebral cortex and the corresponding areas of the contralateral nonischemic cortex.[4,5] Briefly, the core was defined as the lateral cortex directly underlying the occluded portion of the middle cerebral artery. The intermediate zone was the ring of brain tissue that surrounded the core, whereas the outer zone was the remaining cortical tissue. The tissue samples were weighed with 0.0001 mg precision to obtain each wet weight (W). Samples were then dried in an oven (Blue M Electric Co., Blue Island, IL) at 100°C for 24 hr and reweighed to obtain their dry weight (D). The water content was expressed as percentage wet weight and was calculated as $(W - D)/W \times 100$. The dehydrated section was digested in 1 ml of 1 N nitric acid for 1 week. Then a 0.2 ml aliquot was removed and diluted to 2 ml with deionized water and 3 mM $CsCl_2$ solution. The Na^+ and K^+ contents were measured with a sample of this solution by atomic absorption spectroscopy (IL943TM Automatic Flame Photometer, Instrumentation Laboratory, Inc., Lexington, MA). Flame conditions and detection wavelengths were optimized for sensitivity and linearity. The Cl^- content was measured by a digital chloridometer (Haakebuchler Instruments, Inc., Saddleblock, NJ).

Plasma Glucose

Plasma glucose was determined using a Glucometer II (Miles Laboratories, Inc., Elkhart, IN).

Statistical Analysis

All of the data were expressed as the mean ± SE. Statistical differences among groups were determined using ANOVA and the Dunnett *t* test. Probability values of <5% were considered significant.

RESULTS

Physiological and Pharmacological Parameters

Animals were excluded from this study if their physiological parameters (with the exception of plasma glucose) were not in the normal range during the experiment. ANOVA failed to reveal a difference between the groups in every parameter measured (mean arterial blood pressure, rectal temperature, arterial pH, pCO_2, pO_2, HCO_3^-, and hematocrit) except plasma glucose. The group of nonfasting animals treated with 2 g/kg of ethanol had a greater plasma glucose level than the nonfasting controls. All animals had normal pre- and postischemia blood gases (pO_2, pCO_2, and HCO_3^-), pH, hematocrit, plasma osmolarity, and rectal temperatures. Mean arterial blood pressure was normal before and after MCAO, with no difference among the various groups.

CBF

Introduction of the suture to occlude the blood supply to the territory of the left middle cerebral artery produced an equal fall (~12% of control) in relative surface blood flow in all three groups. The mean percentage of baseline CBF in the contralateral hemisphere was comparable, remaining ~100% in all three groups. There were no significant differences in comparable hemispheric CBF among the three ischemic groups ($p > 0.05$).

Changes in Water and Ion Content

The changes in water content in the core, intermediate, and outer zones of ischemic cortex of all three groups are illustrated in Fig. 1. The water content in the contralateral normal hemisphere of all experimental groups was similar (~78 to 79%). However, in the ischemic hemisphere, the nonfasted control group had a water content of 81.1 ± 0.4% (core), 81.0 ± 0.4% (intermediate zone), and 80.3 ± 0.2% (outer zone). These increases in water in the ischemic

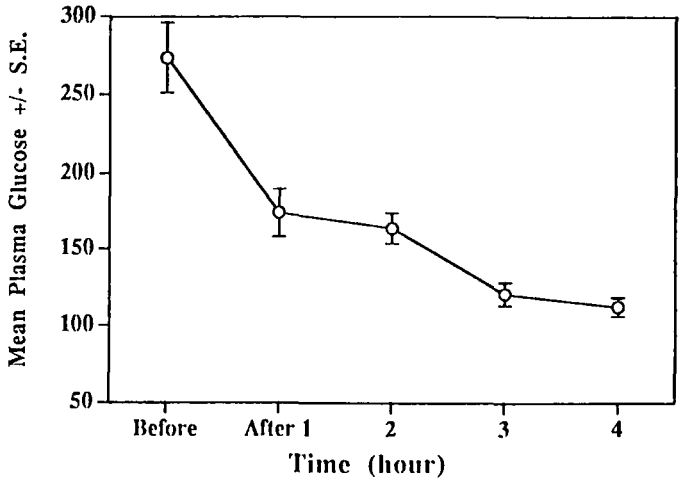

Fig. 2. Plasma glucose levels in ethanol-treated rats given insulin during middle cerebral occlusion. Immediately before intravenous insulin therapy in a dose of 0.25 to 0.5 units/kg, the nonfasted 2 g/kg ip ethanol rats had severe hyperglycemia which, when treated with insulin, were normoglycemic by the end of the 4 hr period of MCAO. In this and Fig. 3, plasma glucose concentrations are in mg/dl.

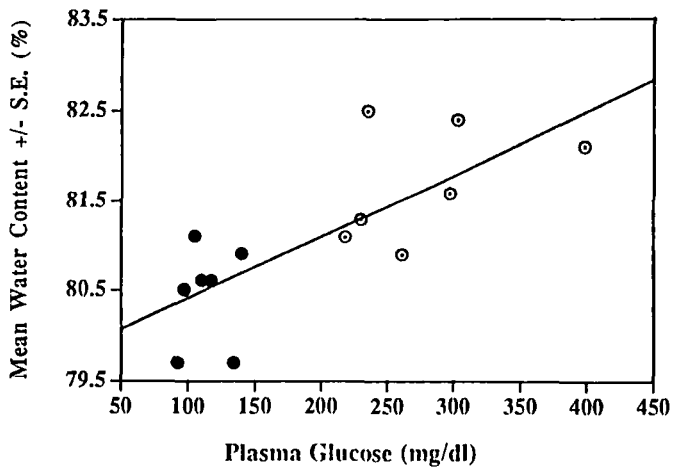

Fig. 3. Relationship between plasma glucose concentration and brain edema. Plasma glucose levels of the control nonfasted (●) and the 2 g/kg ethanol nonfasted (⊙) rats are plotted on the x-axis versus the mean percentage of water content of the core region on the y-axis. There is a significant correlation ($r = 0.76$, $p < 0.05$).

hemisphere were statistically significantly greater than in the nonischemic cortexes ($p < 0.05$). The water content in the ischemic hemisphere of the nonfasted animals treated with 2 g/kg of ethanol was also significantly increased ($p < 0.05$), compared with the control ischemic group. The third group of nonfasted animals treated with 2 g/kg of ethanol were given insulin (0.25 to 0.5 units/kg, iv) to maintain plasma glucose levels normal. In this group, brain edema in the ischemic areas was similar to the control ischemic animals ($p > 0.05$). The water gain of the ischemic brain tissue was accompanied by comparable shifts in Na^+, K^+, and Cl^-.

Correlation between Brain Edema Formation and Plasma Glucose during MCAO

One group of nonfasting rats was treated with regular insulin in incremental doses of 0.25 units/kg iv within 3 min after occlusion, and another dose as needed to maintain a normal plasma glucose level. The plasma glucose level before and after insulin during MCAO is shown in Fig. 2. Before MCAO, the plasma glucose was 287 ± 28 mg/dl. After administration of 0.25 to 0.5 units/kg iv of insulin, the glucose concentration progressively decreased and was in the normal range after 4 hr. Correlation between water content in the ischemic core and levels of plasma glucose is shown in Fig. 3 ($r = 0.76$; $p < 0.05$). Edema formation was significantly less in the rats treated with insulin ($p < 0.05$) that resulted in normal glucose blood levels.

DISCUSSION

A confounding variable in the present in vivo studies is that isoflurane, oxygen, and air are used to produce general anesthesia for the surgical procedure of MCAO. Thus, one is studying the effects of ethanol plus general anesthesia, compared with the effects of general anesthesia alone on brain ischemia. General anesthesia must be used for ethical reasons. Until more reliable methods are developed for producing MCAO without anesthesia, this variable must be present in research of this type. Depth of anesthesia (with and without the added ethanol) was closely monitored using arterial blood pressure, relief of surgical pain as noted by reflex motor movements, normal arterial blood gases, etc. A period of 4 hr of ischemia was used because such a period of MCAO is known to produce statistically significant increases in brain edema in control ethanol free animals. Furthermore, a small amount of 5% glucose was used as the vehicle to make certain that the animals were normoglycemic before ethanol administration. Inasmuch as ethanol induces hypothermia, it was very important to maintain normal body temperature, which was measured rectally. It is known that brain temperature is slightly lower than body temperature in MCAO surgically prepared animals, but this was consistent across all of the groups studied. A brain probe to measure brain temperature directly might cause some brain damage and, therefore, was avoided.

One mechanism by which in vivo ethanol worsens brain edema is to enhance blood glucose levels in nonfasted animals. When a group of similarly treated animals were given sufficient insulin to maintain normal glucose levels, ethanol (2 g/kg, ip) did not increase brain edema. There is a large literature on the beneficial effects of reduced carbohydrate diet, weight loss, insulin, and mild hypoglycemia on acute brain damage, and the opposite with hyperglycemia.[6–20] Regional CBF is also decreased during hyperglycemia.[21,22] On the other hand, hyperglycemia reduces thrombotic infarction induced photochemically in rat parietal cortex.[23] However, hyperglycemia is usually neurotoxic in vivo. The purpose of the present study was to design an

experiment that would mimic an in vivo human situation involving relatively large doses of ethanol in which hyperglycemia would probably occur. This was accomplished using nonfasting ethanol-treated rats. As predicted, the control nonfasting ischemic rats had the same degree of edema formation as nonfasting + ethanol (2 g/kg) and insulin-treated animals. One possibility is that the hyperglycemic action of ethanol in fed rats caused the enhanced edema formation. Insulin attenuates ischemic brain damage independent of its hypoglycemic effect.[16] Hence, it may be that insulin alone was brain protective. In a separate study, we have shown that 2 g/kg ip of ethanol in fasted rats that are normoglycemic does not enhance brain edema. Therefore, one can conclude that hyperglycemia induced by the 2 g/kg dose of ethanol is the mechanism of acute neurotoxicity. However, fasting rats given a larger dose (3 g/kg) of ethanol before induction of ischemia do not have hyperglycemia, but have more severe brain edema compared with control nonethanol-treated animals.[24] Thus, there is still another mechanism, unrelated to ethanol-induced hyperglycemia, for the deleterious effects of ethanol that remains to be clarified. Ethyl alcohol-induced hyperglycemia is mediated in part via catecholamine release. The mechanism of hyperglycemic brain injury remains to be elucidated, but may involve lactic acidosis and endothelial cell injury.[25] The results of the present study indicate the importance of maintaining normoglycemia in investigations of ethanol-induced neurotoxicity in vivo. Future studies should also determine, in addition to ethanol-induced brain edema, specific neuronal damage, as well as survival and functional outcome with and without insulin therapy.

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