Hyperammonaemia depresses glucose consumption throughout the brain

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1. Recent studies showed that hyperammonaemia caused many of the metabolic changes in portacaval-shunted rats, a model of hepatic encephalopathy. These changes included a depression in the cerebral metabolic rate of glucose (CMR_{Glc}), an indication of decreased brain function. 2. The purpose of the present experiments was to determine whether the depression of CMR_{Glc} caused by ammonia is confined to certain brain structures, or whether the depression is an overall decrease in all structures, such as occurs in portacaval-shunted rats. To accomplish this objective, rats were made hyperammonaemic by giving them intraperitoneal injections of 40 units of urease/kg body wt. every 12 h; control rats received 0.154 M-NaCl. CMR_{Glc} was measured 48 h after the first injection, by using quantitative autoradiography with [$6\text{-}^{14}\text{C}$]glucose as a tracer. 3. The experimental rats had high plasma ammonia concentrations (control 70 nmol/ml, experimental 610 nmol/ml) and brain glutamine levels (control 5.4 $\mu\text{mol/ml}$, experimental 19.5 $\mu\text{mol/ml}$). Hyperammonaemia decreased CMR_{Glc} throughout the brain by an average of 19%. CMR_{Glc} showed an inverse correlation with plasma ammonia, but a stronger correlation with the brain glutamine content. 4. Hyperammonaemia led to a decrease in CMR_{Glc} throughout the brain that was indistinguishable from the pattern seen in portacaval-shunted rats. This is taken as further evidence that the cerebral depression found in portacaval-shunted rats is a consequence of hyperammonaemia. The observation that depression of CMR_{Glc} correlated more closely with brain glutamine content than with plasma ammonia suggests that metabolism of ammonia is an important step in the pathological sequence.

INTRODUCTION

The diminution of cerebral function that occurs in human patients with liver disease and hyperammonaemia is mirrored by lower cerebral metabolic rates of glucose (CMR_{Glc}) and oxygen (Hawkins & Mans, 1989a). Portacaval shunting in rats, a common model of chronic liver dysfunction, also results in hyperammonaemia and a substantial decrease in the CMR_{Glc} (Mans *et al.*, 1983, 1986). This decrease begins its downward course within 6 h of shunting and is established by 1 or 2 days (DeJoseph & Hawkins, 1991; Mans *et al.*, 1990). Ammonia is suspected to be responsible for causing cerebral dysfunction, although the mechanism remains unknown (Plum & Hindfelt, 1976; Butterworth *et al.*, 1987; Cooper & Plum, 1987). In recent studies on whole brain it was found that cerebral dysfunction, as indicated by a depression in CMR_{Glc} , could be caused by hyperammonaemia in the absence of liver disease (Jessy *et al.*, 1990). The purpose of the present experiments was to determine where in the brain the decrease in CMR_{Glc} takes place, and to compare the pattern of changes with those known to occur after portacaval shunting. To accomplish this, rats were made hyperammonaemic by giving them several intraperitoneal injections of urease (Jessy *et al.*, 1990) over a 48 h period and measuring regional CMR_{Glc} with [$6\text{-}^{14}\text{C}$]glucose and quantitative autoradiography (Hawkins *et al.*, 1985).

MATERIALS AND METHODS

Materials

D-[$6\text{-}^{14}\text{C}$]Glucose (2030 kBq/ μmol) was bought from New England Nuclear, Boston, MA, U.S.A. X-ray film (Lo Dose/SR329) was obtained from Dupont De Nemours & Co.,

Wilmington, DE, U.S.A. Crystalline jack-bean urease (catalogue no. U0251) and glutaminase were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Reagents and chemicals used were of the best available grade.

Rats

Adult male Long-Evans rats were bought from Charles River Laboratories, Wilmington, MA, U.S.A. All rats were acquired, cared for and handled in conformity with the U.S. Public Health Service's 'Guide for the Care and Use of Laboratory Animals' (NIH Publication No. 86-23, revised 1985) and the 'Guiding Principles for Research Involving Animals and Humans' (recommendations from the Declaration of Helsinki) approved by the Council of the American Physiological Society. They were maintained at 20 °C with 12 h-light/12 h-dark cycles. Food and water were freely available until the time when CMR_{Glc} was measured. The rats weighed 302 ± 39 (S.D.) g at the time the experiments began.

Assays

Glucose, ammonia and glutamine were measured by enzymic methods (Kirsten *et al.*, 1963; Bergmeyer, 1974).

Treatment of rats

The rats were given four intraperitoneal injections of urease (40 units/kg body wt.), one injection every 12 h (1 unit of urease produces 1 μmol of NH_3 from urea/min at pH 7 and 25 °C). CMR_{Glc} was measured 12 h after the last injection (48 h after the first injection). The controls received injections of 0.154 M-NaCl. The body weight was recorded before beginning the urease injections and again before measuring CMR_{Glc} .

Abbreviation used: CMR_{Glc} , cerebral metabolic rate of glucose.

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Measurement of glucose consumption

The measurement of CMR_{Glc} by quantitative autoradiography is relatively straightforward. The essential requirement is a tracer of glucose metabolism that generates a labelled product which remains at the site of metabolism throughout the measurement period. There are two distinctly different types of tracers currently being used: fully metabolizable glucose labelled in a specific position, for example $[6-^{14}C]$ glucose, and glucose analogues such as 2-deoxy-D- $[^{14}C]$ glucose. In our laboratory we prefer the use of $[6-^{14}C]$ glucose because the duration of the experiment can be minimized and the kinetic considerations are simpler. The calculation of CMR_{Glc} by using $[6-^{14}C]$ glucose as a tracer was in accordance with Hawkins *et al.* (1985). The elements of the calculation may be summarized as:

$$CMR_{Glc} = Cm^*/\phi$$

Cm^* , which is determined from the autoradiographs, refers to the net accumulation of ^{14}C in metabolites within a brain region. Cm^* is calculated by measuring the total tissue radioactivity in a given structure and subtracting the amount of $[6-^{14}C]$ glucose remaining unphosphorylated. (The unphosphorylated $[6-^{14}C]$ glucose is calculated from the plasma glucose and the brain/plasma glucose ratio by using exchange constants determined in other experiments.) ϕ refers to the integral of brain glucose specific radioactivity. It is calculated from the plasma glucose specific-radioactivity values that are measured directly.

Preparation of rats and conduct of the CMR_{Glc} measurement

CMR_{Glc} was measured in one rat per day. The rats were anaesthetized with halothane (induction with 4% halothane in air, maintenance with 1–1.5% halothane in N_2O/O_2 , 7:3). Catheters were placed in a femoral artery and vein within 20 min, and the wound was treated with lidocaine. After anaesthesia was discontinued, the rats were placed in a restraining cage for 1 h. Recovery from anaesthesia occurred within minutes, and it is expected that halothane and N_2O were eliminated by the time CMR_{Glc} was measured. Body temperature was monitored and maintained at 37 °C throughout the experiment.

Heparin (200 units) was given 15 min before measuring CMR_{Glc} , and a sample of arterial blood (500 μ l) was drawn for determination of plasma glucose, ammonia and amino acids. The measurement of CMR_{Glc} started with a single intravenous injection of $[6-^{14}C]$ glucose (4.3 MBq/kg) in 0.154 M-NaCl. Blood samples (100 μ l) were taken for determination of glucose specific radioactivity at 0.5, 1, 2, 3 and 4.5 min. A cardioplegic dose of pentobarbital sodium (150 mg in 1 ml of 0.154 M-NaCl), which stopped the heart within 5 s, was used to kill the rats at 5 min. They were decapitated 6–8 s after the pentobarbital injection.

Brain/plasma glucose ratio

This ratio is required for computing the exchange constants (k_2 and k_3) that are necessary for calculating the brain glucose specific-radioactivity integral as well as the amount of unphosphorylated $[6-^{14}C]$ glucose remaining in brain (Hawkins *et al.*, 1985). The ratio is increased by hyperammonaemia and correlated with brain glutamine (ratio = $0.169 + 0.0103[\text{glutamine}]$; Jessy *et al.*, 1990). Therefore, this relationship was used to calculate the brain/plasma glucose ratio in urease-treated rats. The ratio 0.22 was used for control rats (Hawkins & Mans, 1989b).

Autoradiography

The brain was quickly removed from the skull and frozen between layers of bromobutane and methylbutane chilled to –29.8 °C in liquid dichlorodifluoromethane (Hawkins & Mans,

1989b). The brain was kept under liquid dichlorodifluoromethane at –70 °C until sectioning. Thin sections (50 μ m) were made at –10 °C by using a precision microtome (Slee International, New York, NY, U.S.A.). The sections were mounted on slides, dried, and exposed to X-ray film for 2 weeks. Each film was incubated with a set of $[^{14}C]$ methylmethacrylate standards that allow the conversion of absorbance into d.p.m./g. The absorbances were measured in individual areas about 300 μ m in diameter by using a Tobias densitometer (Tobias, Ivyland, PA, U.S.A.). The sections not used for autoradiography (about 85 % of the brain) were collected and homogenized in 1.2 M-HClO₄. Glutamine was measured in the neutralized supernatant.

Statistical analyses

SAS procedures (Statistical Analysis System; SAS Institute, Cary, NC, U.S.A.) were used for all statistical analyses. The data on regional CMR_{Glc} experiments were analysed by analysis of variance and tests of comparison. Measurements of CMR_{Glc} in different brain regions were not considered to be entirely independent. The data were therefore arranged so that the rate values in the different areas were considered to be multiple measurements in each rat, resulting in a mixed model nested ANOVA design. Differences were considered to be significant at $P < 0.05$.

The degree of interaction, which reveals whether the structures were affected by the treatment in a similar manner, was determined as follows. The brain was divided into five major divisions: telencephalon, diencephalon, mesencephalon, metencephalon and myelencephalon. The experimental and control groups were compared by using a repeated-measures analysis of variance. The interaction within each major division was evaluated by the Hyunh–Feldt test.

The average values of CMR_{Glc} for whole brain (average of all areas) and for the groups listed above were analysed by ANOVA to determine whether there was a significant effect of hyperammonaemia. Linear regression procedures were used to evaluate the relationships of ammonia and glutamine with CMR_{Glc} .

RESULTS

Physiological variables

Blood glucose levels were normal in the urease-treated rats (Table 1). Urease treatment caused a small decrease in body wt. of about 8 %. The liver/body-wt. ratio was normal. The ammonia concentration in plasma rose to an average of about 8 times the normal value, and brain glutamine was about 4 times higher. The plasma ammonia and brain glutamine values showed considerable variation in both control and urease-treated rats.

Table 1. Physiological variables in control and urease-treated rats

Values are expressed as means $\pm$ S.E.M., with the number of experiments in parentheses; * $P < 0.05$. All variables were measured after 48 h of hyperammonaemia caused by intraperitoneal injections of 40 units of urease/kg body wt. every 12 h.

Variable	Control (11)	Urease-treated (10)
Plasma glucose (μ mol/ml)	6.99 $\pm$ 0.59	8.551.15
Plasma ammonia (nmol/ml)	70 $\pm$ 6	610 $\pm$ 63*
Brain glutamine (μ mol/g)	5.44 $\pm$ 0.67	19.6 $\pm$ 2.2*
Change in body wt. (%)	+ 5.53 $\pm$ 0.6	– 7.89 $\pm$ 0.7
Liver/body-wt. ratio (%)	4.10 $\pm$ 0.13	3.85 $\pm$ 0.16

Table 2. Regional glucose consumption in brain of hyperammonaemic rats

Results are given as means $\pm$ S.E.M. ($\mu\text{mol}/\text{min}$ per 100 g), with the number of experiments in parentheses: * $P < 0.05$. Regional brain glucose consumption was measured after 48 h of hyperammonaemia caused by intraperitoneal injections of 40 units of urease/kg body wt. every 12 h. $\Delta\%$, % difference between control and urease-treated rats. Abbreviation: n, nucleus.

	Control (11)	Urease- treated (10)	$\Delta\%$
Telencephalon			
Cortex			
olfactory	132 $\pm$ 7	100 $\pm$ 11	-24*
claustrum	139 $\pm$ 7	117 $\pm$ 5	-16*
frontal	127 $\pm$ 4	104 $\pm$ 7	-18*
insular	125 $\pm$ 6	110 $\pm$ 9	-12*
piriform	91 $\pm$ 4	80 $\pm$ 7	-12*
cingulate	137 $\pm$ 5	115 $\pm$ 7	-16*
parietal	134 $\pm$ 6	115 $\pm$ 7	-14*
auditory	157 $\pm$ 6	127 $\pm$ 9	-19*
occipital	117 $\pm$ 6	82 $\pm$ 8	-30*
Accumbens n.	93 $\pm$ 6	72 $\pm$ 8	-23*
Caudate	105 $\pm$ 4	89 $\pm$ 6	-15*
Globus pallidus	65 $\pm$ 3	54 $\pm$ 6	-17*
Amygdala	91 $\pm$ 3	74 $\pm$ 7	-19*
Hippocampus			
anterior	80 $\pm$ 4	65 $\pm$ 6	-19*
posterior	81 $\pm$ 3	63 $\pm$ 5	-22*
dentate gyrus	93 $\pm$ 3	78 $\pm$ 6	-16*
Septal n.			
lateral	72 $\pm$ 3	55 $\pm$ 6	-24*
medial	83 $\pm$ 6	62 $\pm$ 8	-25*
Diencephalon			
Habenula	142 $\pm$ 6	116 $\pm$ 6	-18*
Hypothalamus	96 $\pm$ 5	79 $\pm$ 6	-18*
Supraoptic n.	104 $\pm$ 5	78 $\pm$ 6	-25*
Thalamus			
anterior n.	140 $\pm$ 7	118 $\pm$ 6	-16*
ventral n.	123 $\pm$ 4	103 $\pm$ 7	-16*
mediodorsal n.	116 $\pm$ 5	90 $\pm$ 7	-22*
laterodorsal n.	138 $\pm$ 4	115 $\pm$ 7	-17*
medial geniculate	146 $\pm$ 5	125 $\pm$ 6	-14*
lateral geniculate dorsal	133 $\pm$ 4	110 $\pm$ 6	-17*
lateral geniculate ventral	125 $\pm$ 4	102 $\pm$ 7	-18*
gelatinous n.	123 $\pm$ 4	106 $\pm$ 6	-14*
Mammillary n.	147 $\pm$ 9	128 $\pm$ 9	-13*
Mesencephalon			
Substantia nigra	90 $\pm$ 3	71 $\pm$ 7	-21*
Red n.	117 $\pm$ 4	98 $\pm$ 6	-16*
Oculomotor complex	132 $\pm$ 6	108 $\pm$ 6	-18*
Interpeduncular n.	148 $\pm$ 5	126 $\pm$ 7	-15*
Anterior pretectal area	137 $\pm$ 5	115 $\pm$ 5	-16*
Reticular formation	98 $\pm$ 3	78 $\pm$ 5	-20*
Superior colliculus			
superficial grey	130 $\pm$ 5	115 $\pm$ 6	-12*
intermediate grey	125 $\pm$ 5	106 $\pm$ 6	-15*
Inferior colliculus			
external n.	208 $\pm$ 6	162 $\pm$ 7	-22*
central n.	113 $\pm$ 7	90 $\pm$ 5	-20*
Central grey	105 $\pm$ 4	81 $\pm$ 8	-23*
Dorsal tegmental n.	137 $\pm$ 4	113 $\pm$ 7	-18*
Metencephalon			
Pons	106 $\pm$ 5	80 $\pm$ 6	-24*
Cerebellum:			
semilunar cruz	98 $\pm$ 4	74 $\pm$ 6	-24*
nodulus	149 $\pm$ 5	114 $\pm$ 6	-23*
uvula	139 $\pm$ 4	111 $\pm$ 6	-20*
tuber	133 $\pm$ 4	103 $\pm$ 8	-23*
folium	108 $\pm$ 4	96 $\pm$ 7	-11*
declive	104 $\pm$ 4	85 $\pm$ 7	-18*
dentate n.	144 $\pm$ 7	116 $\pm$ 9	-19*
vermis	123 $\pm$ 6	93 $\pm$ 7	-24*

Myelencephalon			
Vestibular	182 $\pm$ 3	146 $\pm$ 7	-20*
Cochlear n.	165 $\pm$ 4	141 $\pm$ 7	-15*
Superior olive	175 $\pm$ 5	143 $\pm$ 10	-18*
Inferior olive	120 $\pm$ 5	97 $\pm$ 6	-19*
Spinal n. trigeminal	113 $\pm$ 2	86 $\pm$ 6	-24*
Reticular formation	101 $\pm$ 2	73 $\pm$ 5	-28*

Fig. 1. Brain glucose consumption decreases as a function of plasma ammonia and brain glutamine

The average CMR_{glc} in all areas of the brain was used to represent glucose consumption in the whole brain: $\circ$, control; $\blacktriangle$, urease-treated. Brain glutamine content is a better predictor of brain dysfunction than plasma ammonia. For other details see the Materials and methods section.

Brain energy consumption

Hyperammonaemia decreased CMR_{glc} by an average of 19% (Table 2). The depression occurred throughout the brain; no area of the brain seemed particularly affected in comparison with the others. Statistical analysis (tests of interaction) showed that the pattern of CMR_{glc} values was indistinguishable within all the major brain divisions. Thus all brain structures were affected equally by hyperammonaemia. There was a close correlation between plasma ammonia and brain glutamine ($r^2 = 0.62$, $P < 0.001$; results not shown). An inverse correlation was observed between whole-brain CMR_{glc} and plasma ammonia ($r^2 = 0.30$, $P < 0.001$; Fig. 1). An even closer relationship existed between brain glutamine and CMR_{glc} ($r^2 = 0.62$, $P < 0.001$; Fig. 1).

DISCUSSION

It was shown previously that urease treatment (40 units/kg body wt. every 12 h) raises the levels of plasma ammonia, brain ammonia and brain glutamine to values comparable with those of portacaval-shunted rats (Jessy *et al.*, 1990). In both portacaval-shunted and hyperammonaemic (urease-treated) rats, it was

found that the depression in CMR_{Glc} was fully established within the first 2 days (DeJoseph & Hawkins, 1991; Jessy *et al.*, 1990; Mans *et al.*, 1990). Therefore 2 days of hyperammonaemia was chosen as an appropriate time to observe the effect of ammonia on CMR_{Glc} and to compare the results with those from portacaval-shunted rats.

The urease-treated rats in the present study appeared normal in behaviour and appearance. Although body wt. decreased, the unchanged blood glucose levels and the absence of a nutmeg appearance of the liver (characteristic of glycogen depletion) indicated that the rats were not starving.

In previous experiments, a decrease in whole-brain CMR_{Glc} was found to be caused by 2 days of hyperammonaemia (Jessy *et al.*, 1990). The present results confirm that CMR_{Glc} is decreased by hyperammonaemia and extend those observations to the level of individual structures. Hyperammonaemia causes an overall depression in energy consumption affecting all structures to the same degree. The widespread decrease in CMR_{Glc} is of particular interest, because it suggests that hyperammonaemia impairs the entire central nervous system. It is also very similar to the situation that occurs in liver dysfunction. As mentioned above, portacaval shunting, which causes liver atrophy and hyperammonaemia, results in decreased CMR_{Glc} throughout the brain. This decrease is apparent within 6 h and is fully established by 2 days, and is constant over at least an 8-week period (DeJoseph & Hawkins, 1991; Mans *et al.*, 1990). The pattern of depressed CMR_{Glc} observed with 2 days of hyperammonaemia was indistinguishable from that observed in portacaval-shunted rats (Mans *et al.*, 1983, 1986; De Joseph & Hawkins, 1991). These observations add further support to the view that hyperammonaemia is responsible for the cerebral dysfunction of liver failure.

Although it is clear that hyperammonaemia leads to a depression of CMR_{Glc} , the data suggest that ammonia may act indirectly. The plasma ammonia and brain glutamine concentrations were closely correlated, as might be expected. However, plasma ammonia was not as good a predictor of the depression of CMR_{Glc} as was glutamine (Fig. 1; see also Fig. 2 of Jessy *et al.*, 1990). Similar observations have been made in human patients with liver disease. The glutamine concentrations in cerebrospinal fluid and the concentrations of α -oxoglutarate, a derivative of glutamine, show a closer relationship to the degree of encephalopathy than does ammonia (Hourani *et al.*, 1971; Vergara *et al.*, 1974; Oei *et al.*, 1979). Taken together, these data suggest that the metabolism of ammonia is an important part of the toxic response. This prediction is borne out in the following paper (Hawkins & Jessy, 1991), where it is demonstrated that no direct effect of ammonia is detectable when its incorporation into glutamine is inhibited. Whether glutamine is responsible for the

depression of brain function that occurs during hyperammonaemia, or whether glutamine is linked in a causative manner to, say, another metabolite is open to question. (The possibility of a toxic metabolite was raised by Vergara *et al.* (1974). They thought that α -oxoglutarate, a transamination product of glutamine, may be a toxin. Intraventricular injections of this metabolite apparently showed no discernible adverse effects (Plum & Hindfelt, 1976; Cooper & Plum, 1987).

In conclusion, hyperammonaemia leads to a homogeneous decrease in CMR_{Glc} that is indistinguishable from the pattern seen in portacaval-shunted rats. This is additional evidence that the cerebral depression found after portacaval shunting is a consequence of hyperammonaemia. The inverse correlation between energy metabolism and brain glutamine content suggests that the metabolism of ammonia is an important link in the chain leading to hepatic encephalopathy.

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