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Neurobiological characterization of an azoxymethane mouse model of acute liver failure

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Abstract

Molecular biological approaches continue to lead to the identification of alterations in expression of genes coding for key central nervous system proteins involved in water homeostasis, energy metabolism and neurotransmitter regulation in acute liver failure (ALF). However, studies aimed at elucidating the pathophysiological consequences of these changes in gene expression are impeded by the lack of a suitable mouse model of ALF. A previous report described hepatic pathology characteristic of ALF resulting from the administration of azoxymethane (AOM) in mice [Matkowskyj, K.A., Marrero, J.A., Carroll, R.E., Danilkovich, A.V., Green, R.M., Benya, R.V., 1999. Azoxymethane-induced fulminant hepatic failure in C57BL/6J mice: characterization of a new animal model. Am. J. Physiol. 277, G455–G462]. In a series of experiments to further assess this treatment as an effective model of ALF, the effects of administration of AOM to male C57BL mice on hepatic and cerebral function were studied. With maintenance of body temperature at 37 °C and control of hypoglycemia, mice developed signs of encephalopathy (decreased locomotor activity followed by loss of righting and corneal reflexes) within 16 h of AOM treatment. AOM-treated mice were hyperammonemic, developed spontaneous hypothermia and brain edema. Brain ammonia concentrations were increased to 0.98 ± 0.12 mM at coma stages of encephalopathy. Brain amino acid profiles determined by HPLC were typical of ALF in other species including humans. Mild hypothermia (35 °C) led to significant attenuation of brain edema, ammonia, and amino acid changes. These findings demonstrate that AOM treatment affords a simple, reproducible mouse model of ALF which may be suitable for the study of the effects of gene manipulation on the cerebral complications of ALF.
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Keywords: Azoxymethane; Acute liver failure; Brain edema; Amino acids; Gene expression

Acute liver failure (ALF) may occur following viral infection or as a result of toxic liver injury. Irrespective of its etiology, ALF is associated with serious neurological complications including hepatic encephalopathy, a syndrome characterized by altered mental status that may rapidly progress to stupor and coma. Brain edema resulting in increased intracranial pressure is a second major complication of ALF. If severe, brain edema leads to intracranial hypertension and brain herniation, a common cause of mortality in ALF.

Despite several decades of research, our understanding of the pathogenesis of brain edema and hepatic encephalopathy in ALF remains incomplete. Analyses using the technique of differential display—RT-PCR reveal alterations in the expression of several genes of the central nervous system (CNS) in rats with ALF induced by hepatic devascularisation (Desjardins et al., 2001). Subsequent studies suggest that these changes of expression involve proteins of primary importance for normal CNS function. Examples include the glutamate transporter EAAT-2 (Knecht et al., 1997), the glycine transporter GLYT-1 (Zwingmann et al., 2002), the ‘‘peripheral-type’’ benzodiazepine receptor (PTBR) (Desjardins et al., 1997), the water channel protein Aquaporin IV (Margulies et al., 1999) and glial fibrillary acidic protein (GFAP) (Belanger et al., 2002). Interestingly, most proteins showing altered expression levels as a result of ALF are located on the

Abbreviations: ALF, acute liver failure; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AOM, azoxymethane; CNS, central nervous system; HPLC, high performance liquid chromatography; EAAT-2, excitatory amino acid transporter 2; GLYT-1, glycine transporter 1; GFAP, glial fibrillary acidic protein; GS, glutamine synthetase; PTBR, peripheral-type benzodiazepine receptor; RT-PCR, reverse transcription-polymerase chain reaction

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astrocyte, a finding which is consistent with astrocytic rather than neuronal changes characteristic of ALF.

Assessing the consequences of these changes in gene expression on CNS function in vivo is challenging, particularly in disorders such as ALF where multiple systems are affected. In this regard, the increasing availability of genetically modified animals opens new research avenues that could allow a better understanding of the contribution of such alterations in gene expression in the pathophysiological mechanisms responsible for hepatic encephalopathy and brain edema. Unfortunately, the application of such approaches has been hampered by the lack of an appropriate model of ALF in mice, the species most commonly used for manipulations such as gene knockout or overexpression. A number of hepatotoxic drugs have been used to produce ALF, but with limited success. For example the use of acetaminophen-induced hepatotoxicity is limited due to its lack of reproducibility (Terblanche and Hickman, 1991). Thioacetamide has also been used, but variations in toxicity can occur depending on the age and strain of animals, and very little characterization has been performed with this toxin in mice (Sarhan et al., 1993; Itzhak et al., 1995; Kadota et al., 1996). However, recently, a study by Matkowskyj et al. (1999) reported that azoxymethane (AOM) administration to C57Bl mice may provide a novel model for the study of ALF. In the present study, we describe the hepatic and neurobiological consequences of AOM administration to mice and report the protective effects of hypothermia in AOM-induced liver failure. Brain and blood ammonia levels and amino acids concentrations as well as brain water content were measured in animals maintained normothermic or mildly hypothermic following AOM administration.

1. Experimental procedures

1.1. Animals

Male C57BL mice weighing 25–30 g were obtained from Charles River, Saint-Constant (Qc, Canada) and housed with free access to standard laboratory chow and water under constant conditions of temperature, humidity and light cycling. Mice received a single intraperitoneal injection of AOM (100 μg/g; Sigma, St. Louis, CO) dissolved in 100 μL saline. Control animals received an equivalent volume of saline. Following injection of AOM, body temperature was monitored and maintained at 37 °C by means of heating pads (the AOM 37 °C group). In the AOM 35 °C group, hypothermia occurred spontaneously in the absence of external heating and body temperature was maintained at 35 °C using heating pads when necessary. Tail blood glucose concentration was monitored and maintained within normal range by means of subcutaneous injection of a sterile 10% glucose solution (Baxter, Toronto, Canada). When coma stage of encephalopathy was reached (defined as the loss of corneal reflex), normothermic animals were sacrificed by decapitation and brains and livers were rapidly removed. Hypothermic animals were sacrificed in parallel with normothermic and control groups of animals. Animals were handled in accordance with the Guidelines of Canadian Council of Animal care and protocols were approved by the Animal Research Committee at Saint-Luc Hospital (C.H.U.M.).

1.2. Liver histology

Livers were fixed overnight by immersion in 10% buffered formalin. Parrafin-embedded specimens were prepared and 6 μM sections were mounted on Vectabond-coated microscope slides (Vector, Burlington, Canada). HPS (hematoxylin-phloxin-saffron) coloration was performed according to a standard protocol and liver pathology was assessed by an investigator who was blinded to the experimental treatment groups.

1.3. Brain water measurement

Brains were rapidly cut in 2 mm slices and kept at 4 °C. 1 mm punch biopsy samples were obtained from the grey matter of the cerebral cortex and water content was measured gravimetrically using a density gradient of bromobenzene-kerosene (Fisher Scientific, Pittsburgh, PA) precalibrated with K_2SO_4 as previously described (Marmarou et al., 1978). The cortical samples were placed onto the fluid column, and the equilibration point was measured after 2 min. The specific gravity of the tissue was calculated and results are expressed as percentage of water content. Six measurements were made per animal and values were arithmetically averaged.

1.4. Ammonia measurement

Tail blood ammonia levels were measured using the Ammonia Checker II (AA-4120) (Kyoto Daiichi Kagaku CO, Japan) in conjunction with Ammonia Test Kit strips (Arkray Factory Inc., Japan) according to the manufacturer's protocol. Samples above the detection limit were diluted five-fold before measurement and values were corrected according to the dilution factor. Brain ammonia was measured using an ion exchange method. Immediately after decapitation, the head was flash frozen in isopentane cooled to liquid nitrogen temperature and the brain was subsequently chiselled out with special care to keep it frozen at all times. Brains were stored at −70 °C until analysis at which time frozen brains were weighed and powdered over liquid nitrogen. Frozen powder was extracted by homogenization in ice-cold 1 M perchloric acid and centrifuged at 16,000 × *g* for 15 min at 4 °C. 50 μL of supernatant were added to a suspension of Dowex 50WX8-200 ion exchange resin (Aldrich, WI) and allowed to react for 5 min with constant shaking. After extensive washing, the colorimetric reaction was initiated by the addition of phenol and hypochlorite in the presence of nitroferricyanide. Absorbance was measured using a spectrophotometer at a wavelength of 630 nm.

1.5. Plasma and brain amino acid measurements

For brain amino acid analysis, frontal cortices were dissected, weighed and homogenized in 1 M perchloric acid. Plasma samples were deproteinized using 1 M perchloric acid. Samples were mixed by vortexing and the precipitate was removed after centrifugation at 16,000 × *g* for 20 min. The supernatant was neutralized using 25% $KHCO_3$ and used for derivatization. Amino acid analyses were performed using the Agilent 1100 Chemstation reverse-phase HPLC system (Agilent Technologies, Germany) with fluorescence detection (excitation at 338 nm and emission at 280–900 nm) and precolumn *o*-phthalaldehyde (OPA; Sigma) derivatization. Sample injection was performed using an autosampler with derivatization accessories. A 15 μL sample was reacted at room temperature with 20 μL OPA reagent in the presence of 2-mercaptoethanol (Sigma) for 45 s. 25 μL of this solution was injected onto the reverse-phase column (BetaBasic C18) fitted with a C18-5 Unigard Column Protection (Thermo Electron Corporation, Waltham, MA). The chromatogram was eluted with a gradient between methanol (Fisher Scientific, Pittsburgh, PA) and sodium phosphate buffer (50 mM, pH 6.2 containing 2% tetrahydrofuran (Sigma)) at a constant flow rate of 2 mL/min. The elution gradient went from 10% to 50% methanol over 40 min, was then held at 50% methanol for another 10 min before re-equilibration with 10% methanol for 10 min. Peak area was determined using an automated integrator (Agilent Technologies, Germany) and individual amino acid concentrations were calculated in relation to internal standards (homoserine and norvaline) using previously established standard curves.

1.6. Statistical analysis

Results are expressed as mean ± S.D. Comparison of values was analyzed by one-way analysis of variance (ANOVA). Differences between groups were evaluated by the post-hoc Tukey Test. A p value < 0.05 was considered to indicate a significant difference.

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