Reduced brain Na\(^{+}\),K\(^{+}\)-ATPase activity in rats with galactosamine-induced hepatic failure: relationship to encephalopathy and cerebral oedema

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Summary

1. Previously we have shown that sera from patients with fulminant hepatic failure (FHF) will inhibit partially purified rat brain Na\(^{+}\),K\(^{+}\)-ATPase and sodium efflux from human leucocytes in vitro. Similar inhibition may be involved in the pathogenesis of encephalopathy and cerebral oedema in these patients.

2. In the present study we have attempted to establish whether the activity of brain Na\(^{+}\),K\(^{+}\)-ATPase is decreased in vivo in rats with D-galactosamine induced hepatic failure using homogenates of snap-frozen brains.

3. Na\(^{+}\),K\(^{+}\)-ATPase activity was significantly reduced in the forebrain region at the stage of mild encephalopathy (43 h after injection), while at the deeper stage of coma (43–53 h after injection) enzyme activity was further reduced in the forebrain region and was also significantly reduced in the hindbrain region. Ouabain insensitive ATPase activity was not significantly altered at any time.

4. While a significant increase in the water content (0.5%) of the hindbrain region was found 43 h after galactosamine, there was no clear correlation between the development of cerebral oedema and the reduction of Na\(^{+}\),K\(^{+}\)-ATPase activity.

5. The activity of partially purified normal rat brain Na\(^{+}\),K\(^{+}\)-ATPase was 15% lower when incubated with sera from rats in the deep stage of coma compared with control sera.

6. These data support other evidence that the reduction in brain Na\(^{+}\),K\(^{+}\)-ATPase is likely to be due to toxic substance circulating in serum which have been shown to inhibit this enzyme in vitro and to cause coma when administered to normal animals.

Key words: cerebral oedema, encephalopathy, galactosamine, liver failure, Na\(^{+}\),K\(^{+}\)-ATPase.

Abbreviations: BBB, blood–brain barrier; FHF, fulminant hepatic failure.

Introduction

The pathological mechanisms underlying the development of encephalopathy and the associated cerebral oedema in fulminant hepatic failure (FHF) are imperfectly understood. There is, however, considerable evidence that toxic substances accumulating in the circulation play an important part. Thus ammonia, phenols, mercaptans, short chain fatty acids and endotoxin have been shown to increase blood–brain barrier (BBB) permeability [1] and to have synergistic effects in producing coma [2, 3] in normal animals. Animals with experimentally induced acute liver failure show similar increases in BBB permeability [4]. Many of these same substances have also been shown to inhibit the activity of Na\(^{+}\),K\(^{+}\)-ATPase in vitro [5–7]. Reduced activity of this enzyme is associated with an increase in intracellular sodium and impaired neurotransmission as well as being a characteristic feature of the cytotoxic form of cerebral oedema [8].

In recent studies from this Unit we demonstrated that sera from patients with FHF reduced the activity of a partially purified rat brain Na\(^{+}\),K\(^{+}\)-ATPase preparation in vitro [9, 10]. In the present
study we have investigated the relationship in vivo between brain Na\(^{+},K^{+}\)-ATPase activity and the development of encephalopathy and cerebral oedema in the \(\beta\)-galactosamine hepatitis model of FHF in the rat. Cerebral changes in this animal model closely reflect those occurring in man including the development of cerebral oedema [11].

Materials and methods

Experimental animals

Male Wistar albino rats weighing 180–210 g from the stock colony of the animal house at King's College School of Medicine and Dentistry were used throughout. The animals were housed in groups of two or three and were maintained at a temperature of 20°C (range 18–22°C). All animals were allowed free access to food (Labsure diet, CRM, Croydon, U.K.) and water. \(\beta\) (-\(+\) )-Galactosamine hydrochloride (Sigma Chemical Co., Poole, Dorset, U.K.) was injected intraperitoneally in a dose of 2.5 g/kg. The galactosamine was dissolved in sterile water and adjusted to pH 7.3 with 5 mol/l sodium hydroxide immediately before injection. Control rats received an equivalent volume of normal saline (150 mmol/l NaCl, 1 ml/200 g). Twenty-four hours after galactosamine injection the drinking water was replaced by 5% glucose to prevent hypoglycaemia. Control animals received water throughout. Animals were studied in batches of four to ten and in each batch litter-mates were used as controls.

Mortality and time-course studies

The overall mortality for this animal model was determined in 16 rats followed to death or recovery. Eleven (69%) of the 16 rats died, ten doing so between 43 and 60 h after galactosamine injection. In separate studies the time-course of the hepatitis was determined in animals studied at 30, 40 and 50 h after injection of galactosamine and for comparison control rats were studied at similar intervals. Immediately before killing the rats were weighed, stunned and a blood sample taken by cardiac puncture for biochemical determination of liver function.

Brain water content and ATPase activity

The rats were studied in two groups. In the first group 39 animals were killed at 43 h after galactosamine injection when some of them were drowsy (immobile unless stimulated), but none was in deep coma. In the second group ten animals were observed continuously and the seven animals which developed deep coma (as evidenced by immobility with little or no response to physical stimulation and decreased or absent righting reflexes) were weighed and killed. All rats were killed by decapitation, the brains rapidly removed and divided into fore- and hind-brain regions by a transverse incision between the cerebrum and cerebellum. The portions were further divided into right and left halves and the right fore- and hind-brain regions, which were snap-frozen in liquid nitrogen within 90 s of decapitation, were stored at \(\sim 70^\circ\)C for subsequent determination of ATPase activity. The left fore- and hind-brain portions were dried in an oven at 110°C to constant weight.

Serum separated from a blood sample taken at the time of death was stored at \(-20^\circ\)C for determination of liver function tests using standard techniques on a multi-channel analyser (SMAC, Technicon Instruments, Basingstoke, Hants., U.K.). Blood glucose was estimated at the point of death using B.M. Stix (Boehringer, Lewes, Sussex, U.K.) or an enzyme-linked spectrophotometric method [12]. Both methods were shown to be comparable. The Normotest blood clotting time [Nyegaard and Co., Oslo, Norway, supplied by BDH Chemicals Ltd, Poole, Dorset, U.K.], which is sensitive to coagulation factors II, VII and X, was determined to confirm the development of severe hepatic failure. A 10 \(\mu\)l capillary blood sample was taken from the tail immediately before killing, and the clotting time measured with a maximum time limit of 660 s.

ATPase assays

The snap-frozen individual brain regions (see above) were homogenized in 4 vol. of ice-cold sucrose solution (9.25%, w/v). ATPase activity was determined by a radiometric method essentially as described by Bais [13]. The reaction mixture consisted of 0.05 ml of assay buffer and 0.2 ml of brain homogenate, and 0.2 ml of distilled water or 0.2 ml of 10 mmol/l ouabain to determine the total and ouabain insensitive activity respectively. The final composition of the assay mixture was: Tris–HCl 50 mmol/l, KCl 20 mmol/l, NaCl 100 mmol/l, MgCl\(_2\) 5 mmol/l and EDTA 5 mmol/l. After a 20 min preincubation at 37°C the reaction was started by addition of 0.05 ml of 50 mmol/l \([\gamma\text{-}^{32}\text{P}]\text{ATP}\) (specific activity 1–10 kBq/\(\mu\)mol) to achieve a final concentration of 5 mmol/l. The reaction was terminated after 2 min by the addition of 0.5 ml of charcoal/HCl suspension (250 mg of charcoal/ml of 1 mol/l HCl). After centrifugation the liberated \(^{32}\text{P}\) was determined in the clear supernatant by Cerenkov counting [14]. \(\text{Na}^{+},\text{K}^{+}\)-ATPase was calculated from the difference between total and ouabain insensitive ATPase activity. ATPase activities were expressed as \(\mu\)mol of phosphate released h\(^{-1}\) mg\(^{-1}\)
of protein determined by the method of Lowry et al. [15]. The linearity of the Na⁺, K⁺-ATPase assay was verified for up to 4 min of incubation using volumes of up to 400 μl of brain homogenate. We have shown previously that 5 mmol/l EDTA added to chelate Ca²⁺ ions does not affect the Mg²⁺ activation of the enzyme [10].

In a separate series of experiments the effects on ATPase activity of sera obtained from rats after galactosamine injection were investigated using both the whole brain homogenate in vitro and also rat brain Na⁺, K⁺-ATPase which was partially purified by the method of Ahmed & Judah [16] as used previously [9, 10]. Sera were prepared from blood samples obtained by aortic puncture from normal rats, and from two groups of rats with galactosamine hepatitis, one group being bled at 43 h and the other when the animals had progressed to deep coma (immobility and/or gross ataxia).

Statistical analysis

Significance was determined using Wilcoxon’s rank test or Student’s t-test as appropriate. Results are presented as means ± SEM.

Results

Severe liver damage was already apparent 30 h after d-galactosamine as shown by the greater than eightfold increase in serum aspartate transaminase and the tenfold increase in serum bilirubin (Table 1). By 43 h 24 (62%) of the 39 rats showed some definite neurological abnormality, with drowsiness in 22 and hyper-reactivity in two, while the remaining 15 rats showed no obvious neurological abnormality. The Normotest coagulation time was also significantly prolonged by 43 h with a mean value of greater than 7.5 min as compared with 46 s in the controls. By 50 h some of the rats were already in deep coma and showed little spontaneous activity. Both the mean aspartate transaminase and bilirubin levels showed significant further increases while the mean serum albumin had fallen by 9.7% at 50 h post-galactosamine (Table 1).

Liver and body weight fell progressively during the illness, although the reduction in liver weight was proportionally greater (Fig. 1). At autopsy the livers were shrunken and pale, and by 43 h there were patchy areas of haemorrhage and necrosis in 87%. Although blood glucose concentration was

![Fig. 1. Ratio of liver weight to body weight in control rats (n=22) and at 30 h (n=12), 43 h (n=37) and 50 h (n=18) after intraperitoneal galactosamine. The open circle represents mean time and liver weight to body weight ratio for rats killed after entering deep coma (43–53 h after galactosamine, n=7). Bars represent SEM.](image)

Table 1. Results of Normotest and routine serum biochemical tests of liver function in galactosamine hepatitis and control groups

<table>
<thead>
<tr>
<th>Normotest (s)</th>
<th>Aspartate transaminase (i.u./l)</th>
<th>Alkaline phosphatase (i.u./l)</th>
<th>Albumin (g/l)</th>
<th>Bilirubin (μmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>46 ± 1.4 (12)</td>
<td>288 ± 18 (22)</td>
<td>510 ± 17</td>
<td>32.1 ± 1.3</td>
</tr>
<tr>
<td>Galactosamine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>At 30 h</td>
<td></td>
<td>2387 ± 577 (12)</td>
<td>364 ± 31</td>
<td>—</td>
</tr>
<tr>
<td>At 43 h</td>
<td>&gt; 460* (range 95–&gt; 660) (10)</td>
<td>1639 ± 294* (36)</td>
<td>650 ± 30</td>
<td>28.8 ± 0.3*</td>
</tr>
<tr>
<td>At 50 h</td>
<td>&gt; 480*† (range 75–&gt; 660) (18)</td>
<td>3133 ± 741*† (13)</td>
<td>657 ± 48</td>
<td>26.0 ± 0.9 †</td>
</tr>
</tbody>
</table>
slightly reduced 50 h after galactosamine (3.3 ± 0.3 mmol/l, \(n=16\) compared with control 5.0 ± 0.2 mmol/l, \(n=12\)), it did not fall to a level which would cause hypoglycaemic coma.

**Brain ATPase activity and water content after galactosamine**

\(\text{Na}^+\text{,K}^+\text{-ATPase} \) activity was significantly reduced in the forebrain region 43 h after galactosamine when most of the rats were drowsy but none was in deep coma (4.78 ± 0.01 compared with 5.22 ± 0.11 pmol of Pi h\(^{-1}\) mg\(^{-1}\) of protein in the controls, \(P < 0.001\), Fig. 2). There was no significant change in the hindbrain \(\text{Na}^+\text{,K}^+\text{-ATPase} \) activity 43 h after galactosamine (4.38 ± 0.10 compared with 4.48 ± 0.11). When rats were allowed to progress to deeper coma before killing (see the Methods section) there was a further reduction in \(\text{Na}^+\text{,K}^+\text{-ATPase} \) activity in both fore- and hind-brain regions (4.50 ± 0.2, \(P < 0.005\) and 3.86 ± 0.18, \(P < 0.02\), respectively, compared with control). No similar reduction in enzyme activity was observed in rats at 43 to 53 h which did not go into deep coma (forebrain 5.20 ± 0.12, hindbrain 4.75 ± 0.08). There was no reduction in ouabain insensitive activity at any time after galactosamine.

Brain water content was increased by 0.5% in the hindbrain region 43 h after galactosamine (81.22 ± 0.32%, \(n=26\) as compared with 80.77 ± 0.49, \(n=23\) in controls, \(P < 0.05\)) but there was no such increase in the forebrain region at this time (80.70 ± 0.12% as compared with 80.96 ± 0.11 in the controls). Rats studied in deeper coma (43–53 h) showed a significant reduction in water content of the hindbrain (78.08 ± 0.36%, \(n=7\) as compared with controls; 80.77 ± 0.49, \(n=23\); \(P < 0.01\)) and a smaller decrease (< 1%) in forebrain water content (80.06 ± 0.15% as compared with controls 80.96 ± 0.11, \(P < 0.05\)).

**Effects of FHF rat serum on brain ATPase activity**

No significant effects of sera from FHF rats 43 h after galactosamine could be found on the ATPase activities of whole brain homogenates as compared with sera from normal control animals (Table 2). There was a similar lack of effect of these (43 h) sera on partially purified \(\text{Na}^+\text{,K}^+\text{-ATPase} \) from normal rat brains. Sera obtained from rats which had progressed to deep coma (46–53 h) caused a 15% reduction in activity of \(\text{Na}^+\text{,K}^+\text{-ATPase} \) as

**Table 2. Effect of control sera and sera from galactosamine hepatitis rats (43 h) on ATPase activity in whole brain homogenates from normal rats**

<table>
<thead>
<tr>
<th></th>
<th>Forebrain</th>
<th>Hindbrain</th>
<th>Total ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>((\mu\text{mol of Pi h}^{-1}\text{ mg}^{-1}) of protein)</td>
<td>((\mu\text{mol of Pi h}^{-1}\text{ mg}^{-1}) of protein)</td>
<td>((\mu\text{mol of Pi h}^{-1}\text{ mg}^{-1}) of protein)</td>
</tr>
<tr>
<td>Control (no serum)</td>
<td>4</td>
<td>4.54 ± 0.37</td>
<td>4.83 ± 0.27</td>
</tr>
<tr>
<td>Normal rat serum</td>
<td>5</td>
<td>4.73 ± 0.07</td>
<td>4.39 ± 0.12</td>
</tr>
<tr>
<td>Galactosamine rat serum (43 h)</td>
<td>6</td>
<td>5.04 ± 0.36</td>
<td>4.83 ± 0.13</td>
</tr>
</tbody>
</table>

Fig. 2. \(\text{Na}^+\text{,K}^+\text{-ATPase} \) (stippled columns) and ouabain insensitive ATPase (open columns) activities of homogenates of snap-frozen brains from control (\(n=28\)) and galactosamine (2.5 g/kg) treated rats at 43 h (\(n=39\)) and in deep coma (\(n=7\)). Bars represent \(\text{SEM} \). *\(P < 0.001\) vs control; **\(P < 0.005\) vs control; ***\(P < 0.01\) vs 43 h galactosamine rats.
TABLE 3. Effects of serum from control and galactosamine hepatitis rats on partially purified rat brain Na\textsuperscript{+},K\textsuperscript{+}-ATPase from normal rats

Results are means \pm SEM. Statistical significance: \* \( P < 0.02 \) compared with control sera.

<table>
<thead>
<tr>
<th>No. of Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity</th>
<th>Total ATPase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control serum</td>
<td>Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity (( \mu \text{mol of P(h) mg(^{-1}) of protein} ))</td>
</tr>
<tr>
<td>Gallactosamine hepatitis serum (43 h)</td>
<td>6</td>
</tr>
<tr>
<td>Gallactosamine deep coma serum (46–53 h)</td>
<td>5</td>
</tr>
</tbody>
</table>

compared with sera from normal controls \( (P < 0.02) \) (Table 3).

Discussion

We have demonstrated significant reduction of Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity in brain homogenates of rats with encephalopathy due to galactosamine induced acute liver failure, with a greater reduction in animals with deeper encephalopathy. This reduced activity is most likely to be due to effects of circulating toxic substances which have been shown to inhibit Na\textsuperscript{+},K\textsuperscript{+}-ATPase in a variety of body tissues in vitro [17]. It is unlikely that the reduction in Na\textsuperscript{+},K\textsuperscript{+}-ATPase was due to a general loss of protein from the brain or an alteration in the cell composition since ouabain insensitive ATPase activity, largely Mg\textsuperscript{2+}-ATPase, remained constant throughout. High Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity is found in both glial cells and neurons [18] and is essential for the maintenance of the ionic gradients necessary for neurotransmission [19]. Inhibition of Na\textsuperscript{+},K\textsuperscript{+}-ATPase in endothelial cells of the cerebral capillaries may result in impaired function of the BBB as found experimentally in liver failure [4, 20]. In the same model of liver failure in the rat a threefold increase in permeability to inulin (mol. wt. 5000) was found [4] and as the toxic substances appear from gel chromatography to have a mol. wt. lower than this [10] they are likely to enter brain and exert their toxic effects on cerebral neurons.

Reduced activity of this enzyme could also result from changes in the lipid composition [21] of the nerve cell membranes, possibly associated with altered membrane fluidity. Na\textsuperscript{+},K\textsuperscript{+}-ATPase exists in two forms within the brain [18]. One form is the same as that found in cell membranes in other tissues; the other is unique to brain and is concentrated in the synaptosomes. Inhibition of neuronal Na\textsuperscript{+},K\textsuperscript{+}-ATPase causes the release of the neurotransmitter pool including the inhibitory neurotransmitter \( \gamma \)-aminobutyric acid [22]. This effect may be another mechanism whereby reduction in the activity of this enzyme could lead to encephalopathy.

In this study Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity was assayed under optimal conditions of substrate and cofactors. In vivo, where the activity of the enzyme is never maximal due to close regulation by numerous factors including substrate supply, a greater degree of inhibition than 8–14% could occur. Small changes in enzyme activity would alter the balance in coupled membrane transport systems leading to major disruption of cellular transport. However, alterations in Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity may be only one of the multifactorial metabolic events occurring during coma. Direct measurements of Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity or sodium pump activity in intact brains are not possible and separation of cells from the brain would remove any reversible inhibition through dilution during the separation process. Determination of Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity in concentrated homogenates of whole brains, as in the present study, probably provides a reasonable reflection of the situation in vivo since dilution of possible inhibitors is kept to a minimum. Dilution of the brain samples could alter the balance between substances which stimulate Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity (such as insulin [23] which is present at increased concentration during FHF) [24] and those which inhibit it (such as accumulated toxic substances) and this may explain the lack of inhibition found by Ferenci et al. [25]. Pappas et al. [26] found stimulation of Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity by serum from rabbits with galactosamine induced FHF, contradictory to our findings. The stage at which the serum was collected was not given and we only found inhibition with serum taken from rats with deep coma. Species differences could also be an important factor in these effects. Sodium efflux studies using isolated leucocytes have shown the sodium pump activity of intact cells to be inhibited by substances present in FHF serum [27]. Similarly, inhibition of partially purified rat brain Na\textsuperscript{+},K\textsuperscript{+}-ATPase by serum from patients ([10]; Y. Muto, J. Sugihara, T. Nakamura, M. Kato, E. Tomita & O. Hashimoto, personal communication) and rats (this study) with acute liver failure has been demonstrated.

In the present study the reduction in Na\textsuperscript{+},K\textsuperscript{+}-
there was an 8% reduction in activity in the fore-brain region 43 h after galactosamine, once deep coma supervened there was a 14% reduction in activity in both fore- and hind-brain regions. This is similar to the 15% reduction in activity of partially purified rat brain Na\(^+\),K\(^+\)-ATPase in vitro caused by sera taken from animals at the same stage of encephalopathy.

The regional changes in brain water content showed no clear relationship to the progression of encephalopathy or with the changes in brain Na\(^+\),K\(^+\)-ATPase. This finding is in accord with our previous observations using sera from patients with FHF in which the reduction in Na\(^+\),K\(^+\)-ATPase activity in vitro correlated with the degree of encephalopathy but not with the development of cerebral oedema [10]. Groflin & Tholen [11], using this animal model, also found that significant increases in water content were confined to the hindbrain region with an 0.77% increase in water content in the cerebellum and an 0.54% increase in that of the brainstem 42 h after galactosamine. Thus, it appears that the hindbrain region has an increased susceptibility to oedema formation and, although the reason for this remains to be established, it is of interest that in the control animals the Na\(^+\),K\(^+\)-ATPase activity of this region was 14% lower than in the forebrain. Furthermore, the blood supply to the hindbrain region, which originates from the vertebral arteries, may give a lower mean perfusion pressure than that of the forebrain. Thus in the presence of arterial hypotension, which is common in FHF [28], cerebral perfusion pressure could be critically reduced in this part of the brain, so predisposing it to oedema formation. Groflin & Tholen [11] found the water content of the cerebellum had decreased in animals studied 48 h after galactosamine compared with those studied at 42 h, although there was still a small increase above control values. We also found that the brain water content was reduced at a comparable time but to a level below that of the control animals. This finding is most likely explained by a generalized dehydration of the animals during the later stages of severe galactosamine liver damage. It is not known whether this could be overcome by more intensive fluid replacement, which would be technically difficult to perform.

Thus while we were unable to demonstrate a significant relationship between the inhibition of Na\(^+\),K\(^+\)-ATPase and the development of cerebral oedema, we have found inhibition of this enzyme in concentrated brain homogenates during the course of hepatic encephalopathy in this animal model of FHF which probably reflects changes in sodium pump activity in vivo. It is also apparent from our study that the degree of reduction of brain Na\(^+\),K\(^+\)-ATPase activity and the appearance of inhibitory factors in the serum are time dependent.

Acknowledgment

This study was supported by the Medical Research Council.

References

protein containing cation-dependent ATPase. Biochemical and Biophysical Research Communications, 93, 603-613.


