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Increased Na^+,K^+-ATPase activity in the rat brain after meningitis induction by *Streptococcus pneumoniae*

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Barichello T, Generoso JS, Cipriano AL, Casagrande R, Collodel A, Savi GD, Scherer EBS, Kolling J, Wyse ATS. Increased Na^+,K^+-ATPase activity in the rat brain after meningitis induction by *Streptococcus pneumoniae*.

Background: Pneumococcal meningitis is the most severe infection of the central nervous system with a mortality rate up to 20% and an adverse neurological result in up to 50% of survivors. A complicated series of interactions among the host immune response and oxidants seems to be responsible for meningitis associated brain dysfunctions. Na^+,K^+-ATPase is an essential enzyme responsible for generating and maintaining the membrane potential necessary for neural excitability, however, the Na^+,K^+-ATPase activity is altered in several illness;
Objective: The aim of this study is to evaluate the Na^+,K^+-ATPase activity in hippocampus and cortex of the rats submitted to pneumococcal meningitis.
Methods: Animals received 10 µl sterile saline as a placebo or an equivalent volume of *Streptococcus pneumoniae* to the concentration of 5×10^9cfu/ml and were killed at 24, 48, 72 and 96 h after meningitis induction. The brain structures, hippocampus and cortex, were immediately isolated on dry ice and stored at -80°C to analyse Na^+,K^+-ATPase activity.
Results: In the hippocampus, we verified the increase of Na^+,K^+-ATPase activity at 48, 72 and 96 h ($p < 0.05$) and in the cortex at 24 h ($p < 0.05$) after pneumococcal meningitis induction.
Conclusion: The Na^+,K^+-ATPase activity is under the control of a diversity of intracellular messengers that are able to modulate the function of the particular isozymes in a precise way. Furthermore, we verified that pneumococcal meningitis increased the Na^+,K^+-ATPase activity in hippocampus and cortex; this increase can be correlated with a compensatory mechanism in illness pathophysiology.

Keywords: brain; meningitis; Na^+,K^+-ATPase; *Streptococcus pneumoniae*

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Significant outcomes

- The influence of pneumococcal meningitis in Na^+,K^+-ATPase activity.
- Increased Na^+,K^+-ATPase levels may be related to neuronal damage.
- Our study helps to understand better the pathophysiology of pneumococcal meningitis.

Limitations

- Animal model.
- The results in animal models should be interpreted with caution before correlate with the clinic.
- This study evaluates the animal model of adult rats.

Introduction

Pneumococcal meningitis is characterised by an intense inflammation of the meninges (1) affecting pia matter, arachnoid and subarachnoid space; this may result in brain damage in cortical and subcortical structures (2). Patients who died from bacterial meningitis showed apoptosis in hippocampus and cortical necrosis (3). Furthermore, surviving patients may suffer from permanent neurological sequelae such as deafness, blindness, learning impairments, sensorimotor deficits and seizure disorders (4,5). The bacterial growth inside the subarachnoid space initiates a complex immune response (6). A complicated series of interactions among the host immune response, cytokines, chemokine (7), matrix metalloproteinases, proteolytic enzymes and oxidants seems to be responsible for brain dysfunctions associated with meningitis (8). In previous studies, we verified the increased cytokines and chemokine levels (9), increased oxidative damage to proteins and lipid peroxidation in the first 24 h after pneumococcal meningitis induction (10). Moreover the cytokines stimulates reactive oxygen species (ROS) in mitochondria by altering membrane permeability and by inhibiting the electron transport chain, thereby causing mitochondrial damage (11,12) and resulting in the inability to generate energy in the form of adenosine triphosphate (ATP) (13,14). Na^+,K^+-ATPase (E.C.3.6.1.37) is an essential enzyme responsible for generating and maintaining the membrane potential necessary for neural excitability (15), catalysing the activity uptake of K^+ and extrusion of Na^+ at the expense of hydrolysing ATP generated from cellular glycolysis and oxidative phosphorylation, thus generating steep concentrations gradients for these ions (16). In the neurons, where it may use up to 70% of the cell's total energy consumption, the role of the Na^+,K^+-ATPase is crucial for basis metabolic requirements and for the specialised functions of nerve impulse transmission (17). Earlier studies have shown that Na^+,K^+-ATPase activity is altered in ischaemia (18), neurodegenerative diseases (19), with chronic administration of ketamine (20), increased in animal models using lipopolysaccharide (21) and resulted in increased Na^+K^+ pump activity (22) in sepsis also. Furthermore, Na^+,K^+-ATPase activity was inhibited by interleukin-1β (IL-1β) in cardiac myocytes (23) and reduced cerebral cortical cell membrane Na^+,K^+-ATPase activity in meningitis by *Escherichia coli* in the newborn piglet (24). According to Sellner et al. (25), a problem factor that contributes to this insufficient therapeutic in the meningitis treatment is our deficient understanding of the pathogenesis and pathophysiology of the bacterial infection in the central nervous system. Thus, the aim of our study was to evaluate the Na^+,K^+-ATPase activity in hippocampus and cortex of rats submitted to pneumococcal meningitis.

Materials and methods

Infecting organism

Streptococcus pneumoniae (serotype 3) was cultured overnight in 10 ml of Todd Hewitt Broth, diluted in fresh medium and grown to logarithmic phase. The culture was centrifuged for 10 min at ($5000 \times g$) and re-suspended in sterile saline to the concentration of the 5×10^9 cfu/ml. The accuracy of the inoculum size was confirmed by quantitative cultures (26,27). The bacterial concentration of 1×10^3 can be identified in cerebral spinal fluid (CSF) by Gram stain (28).

Animal model of meningitis

Male Wistar rats (250–300 g body weight) from our breeding colony were used for the experiments. All the procedures were approved by the Animal Care and UNESC's Experimentation Committee, Brazil, and followed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996. All the surgical procedures and bacterial inoculations were performed under anesthesia, consisting of an intraperitoneal administration of ketamine (6.6 mg/kg), xylazine (0.3 mg/kg) and acepromazine (0.16 mg/kg) (9,10,29). Rats underwent a cisterna magna tap with a 23-gauge needle. The animals received either 10 µl of sterile saline as a placebo ($n = 7$) or an equivalent volume of *S. pneumoniae* suspension in the meningitis group ($n = 37$). At the inoculation time, animals received fluid replacement (2 ml of saline subcutaneously) and were subsequently returned to their

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cages. Following their recovery from anesthesia, animals were supplied with food and water *ad libitum*. Meningitis was documented by a quantitative culture of 5 µl of CSF obtained by puncture of the cistern magna (9). Animals were decapitated at 24, 48, 72 and 96 h after meningitis induction. The brain structures, hippocampus and cortex, were immediately isolated on dry ice and stored at −80°C to analyse of Na^+,K^+-ATPase activity.

Tissue preparation

For preparation of synaptic plasma membrane and determination of Na+,K+-ATPase activity, hippocampus was homogenised in 10 volumes of 0.32 mM sucrose solution containing 5.0 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 1.0 mM ethylenediaminetetraacetic acid, pH 7.4. Synaptic plasma membranes were prepared according to the method of Jones and Matus (30), with some modifications (31). The homogenate was centrifuged at 1000 $\times g$ for 20 min; the supernatant was removed and centrifuged at 12.000 $\times g$ for 20 min. The pellet was then suspended in hypotonic buffer (5.0 mM Tris–HCl buffer, pH 8.1), incubated at 0°C for 30 min, and applied on a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8 and 1.0 M. After centrifugation at 69.000 $\times$ g for 2 h, the fraction at the 0.8–1.0 M sucrose interface was taken as the membrane enzyme preparation.

Na^+,K^+-ATPase activity assay

The reaction mixture for Na^+,K^+-ATPase activity assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl and 40.0 mM Tris–HCl, pH 7.4, in final volume of 200 ml. The reaction was initiated by addition of ATP to a final concentration of 3.0 mM. Controls were carried out under the same conditions with the addition of 1.0 mM ouabain. Na^+,K^+-ATPase activity was calculated by the difference between the two assays, as described by Wyse et al. (18). Released inorganic phosphate (Pi) was measured by the method of Chan et al. (32). Specific activity of the enzyme was expressed as nmol Pi released per min per mg of protein. All samples were run in duplicates.

Protein measurement

Protein was measured by the Bradford method (33) with bovine serum albumin used as standard.

Statistics

The variables were showed by mean ± SEM of five to six animals in each group. Differences among groups were evaluated using the variance analysis followed by Student–Newman–Keuls *post hoc* test. p-Values <0.05 was considered statistically significant.

Results

Na^+,K^+-ATPase activity was evaluated in hippocampus and cortex from adult Wistar rats submitted by pneumococcal meningitis. Our results showed that meningitis increased in Na^+,K^+-ATPase activity at 48, 72 and 96 h ($p < 0.05$; $F = 14.992$) in the hippocampus after meningitis induction (Fig. 1a); furthermore, in the cortex, there were an increase in Na^+,K^+-ATPase activity only at 24 h ($p < 0.05$; $F = 2.662$) after meningitis induction when compared with the control group (Fig. 1b).

Discussion

Bacterial cell wall components into the CSF are well known as inflammatory host response inducers (8); so, the neuronal injury is mediated by the release

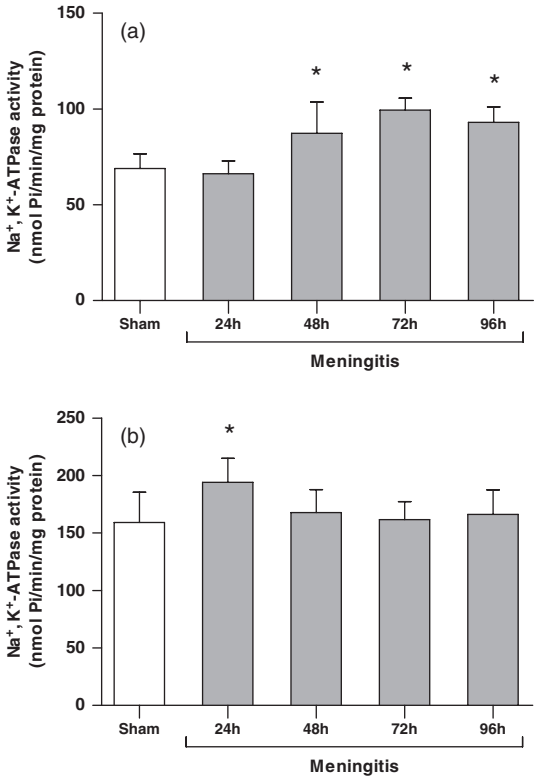

Fig. 1. Na^+,K^+-ATPase activity in hippocampus (a) and cortex (b) after *Streptococcus pneumoniae* meningitis induction. Na^+,K^+-ATPase activity was calculated by released Pi and the specific activity of the enzyme was expressed as nmol Pi released per min per mg of protein. Results show the mean ± SEM of five to six animals in each group. Symbols indicate statistically significant when compared with sham group *$p < 0.05$.

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of ROS, proteases, cytokines and excitatory amino acids, and is executed by the activation of transcription factors, caspases and others proteases (34). In previous studies, we verified that the tumour necrosis factor-alpha levels increased in the first hours and then there was also an increase of the IL-1β, IL-6 and cytokine-induced neutrophil chemoattractant-1 levels (27), lipid peroxidation, protein carbonylation and decreased the protein integrity in hippocampus and cortex after pneumococcal meningitis induction (10). However, in the mitochondrial respiratory chain these levels showed increases in the activities in hippocampus and cortex (35). Na^+,K^+-ATPase is a key regulator of cellular ion homeostasis (16). Our study showed that pneumococcal meningitis increased the Na^+,K^+-ATPase activity in two brain regions, in the hippocampus and cortex of adult rats in different times. In addition, the injury in bacterial meningitis is different in these brain areas and has been characterised by tissue necrosis in the cortical hemispheres and by apoptotic cell death in the hippocampal dentate gyrus (3). In other severe neurological disorders, such as autism, the Na^+,K^+-ATPase activity also increased in the frontal cortex and cerebellum (36). Furthermore, recent studies have reported an association of cytokines with autism (37,38,39) and increased the Na^+,K^+-ATPase activity in response to increased intracellular calcium (36). Lipopolysaccharide also increased Na^+,K^+-ATPase expression in airway epithelium (21). Nevertheless, it is well known that lipopolysaccharide also increases the production of cytokines, chemokines, reactive oxygen and nitrogen species (40). Patients in shock or sepsis have a high production rate of lactic acid that has traditionally been attributed to poor tissue perfusion and hypoxia (41). The increased glycolysis in these situations has been associated to increased activity of membrane ion pumps such as the Na^+,K^+-ATPase (22). McCarter et al. (22) showed that increased muscle lactate during sepsis correlates with evidence of elevated muscle Na^+,K^+-ATPase, but not with the evidence of impaired oxidative metabolism. Furthermore, plasma concentration of the lactate and epinephrine, a known stimulator of Na^+,K^+ pump, were increased in septic rats by *E. coli* (42). Lactic acid is recognised as an important virulence factor for multiple streptococcal species (43) and can be associated with the increase of Na^+,K^+-ATPase activity. Their activity is under the control of a diversity of intracellular messengers that are able to modulate the function of the particular isozymes in a precise way (17). The sodium pump is not simply an ion transporter because the Na^+,K^+-ATPase isozymes have kinetic properties that are single; isozyme-specific regulation may be important in adapting Na^+ pump function to the necessities of each cell (17,44), and it is responsible for generating and maintaining membrane potential and thus disturbances in its activity could have grave consequences for neuronal functioning (16). So, the present findings showed that pneumococcal meningitis increased the Na^+,K^+-ATPase activity; thus, toxic substances and proteases produced by bacteria can cause damage to neurons directly. For example, hemolysin and pneumolysin released by the bacteria are capable of causing damage to neurons, probably by promoting the influx of extracellular Ca^{+2} (34). Na^+,K^+-ATPase activity also increased in response to increased intracellular calcium (36). Our results should be interpreted with caution before correlation with the clinic; however, the rat model allows for a refined assessment of clinical and neurological symptoms (45).

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