NeuroToxicology 54 (2016) 34–43

Contents lists available at ScienceDirect

NeuroToxicology

Full length article

Hyperosmolar sodium chloride is toxic to cultured neurons and causes reduction of glucose metabolism and ATP levels, an increase in glutamate uptake, and a reduction in cytosolic calcium

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ARTICLE INFO

Article history:
Received 7 January 2016
Received in revised form 15 March 2016
Accepted 16 March 2016
Available online 17 March 2016

Keywords:
Hyperosmolarity
Dehydration
Delirium
Neurotoxicity
Sodium
Calcium

ABSTRACT

Elevation of serum sodium, hypernatremia, which may occur during dehydration or treatment with sodium chloride, may cause brain dysfunction and damage, but toxic mechanisms are poorly understood. We found that exposure to excess NaCl, 10–100 mmol/L, for 20 h caused cell death in cultured cerebellar granule cells (neurons). Toxicity was due to Na^+, since substituting excess Na^+ with choline reduced cell death to control levels, whereas gluconate instead of excess Cl^- did not. Prior to cell death from hyperosmolar NaCl, glucose consumption and lactate formation were reduced, and intracellular aspartate levels were elevated, consistent with reduced glycolysis or glucose uptake. Concomitantly, the level of ATP became reduced. Pyruvate, 10 mmol/L, reduced NaCl-induced cell death. The extracellular levels of glutamate, taurine, and GABA were concentration-dependently reduced by excess NaCl; high-affinity glutamate uptake increased. High extracellular [Na^+] caused reduction in intracellular free [Ca^{2+}], but a similar effect was seen with mannitol, which was not neurotoxic. We suggest that inhibition of glucose metabolism with ensuing loss of ATP is a neurotoxic mechanism of hyperosmolar sodium, whereas increased uptake of extracellular neuroactive amino acids and reduced intracellular [Ca^{2+}] may, if they occur *in vivo*, contribute to the cerebral dysfunction and delirium described in hypernatremia.

1. Introduction

An increase in the serum concentration of sodium ions above a physiological concentration, hypernatremia, may cause cerebral dysfunction, including cognitive dysfunction and delirium (for review, see Lieberman, 2007; Pendlebury et al., 2015), cerebral edema (Unal et al., 2008), and destruction of myelin, myelinolysis (Han et al., 2015). Hypernatremia may occur in dehydration, during intravenous infusion of hypertonic saline, or after excessive salt intake (Sterns, 2015), and serum values may in extreme cases exceed 200 mmol/L (Meadow, 1993; Ofran et al., 2004), which is approximately 60 mmol/L above normal serum sodium values. Dehydration may occur in elderly patients and the terminally ill (Bruera et al., 2005; Vidán et al., 2009), and dehydration is a common complication of prolonged fever and of diarrhoea, especially in children (World Health Organization, 2000). Therapeutically, intravenous infusion of hypertonic saline to raise serum sodium by 10–20 mmol/L is used to treat intracranial hypertension (for review, see Himmelseher, 2007; Ropper, 2012). Experimental studies have shown that an increase in the serum concentration of sodium leads to a similar increase in the extracellular fluid of the brain, with a time lag of about 30 min (Cserr et al., 1987, 1991).

In spite of the well described cerebral symptoms of hypernatremia (Lieberman, 2007; Vidán et al., 2009; Sterns, 2015) little is known about the effect of supranormal concentrations of sodium chloride on neuronal function and survival. Himmelseher et al. (2001) found that a 15-min pulse of hypertonic saline and hydroxyethyl starch (350 mOsm/L) was toxic to cultured hippocampal neurons, as could be seen from cell death 24 h later. Bhardwaj et al. (2000) found that hypernatremia aggravated neocortical damage after stroke in rats. Elliott et al. (2007) reported that hypertonic saline given immediately after traumatic brain injury in rats worsened brain damage. In human patients with traumatic brain injury hypernatremia is associated with increased mortality (Alharfi et al., 2013; Li et al., 2013).

Abbreviations: aCSF, artificial cerebrospinal fluid; GABA, γ-aminobutyric acid; LDH, lactate dehydrogenase; NMDA, *N*-methyl-D-aspartate; TCA cycle, tricarboxylic acid cycle.

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http://dx.doi.org/10.1016/j.neuro.2016.03.005
0161-813X/

The aim of this study was to establish whether prolonged exposure of neurons to hyperosmolar NaCl is toxic in an *in vitro* model and to gain insight into the cellular responses of neurons to high concentrations of NaCl, including effects on energy metabolism, handling of extracellular amino acids, and intracellular free [Ca^{2+}].

2. Experimental procedures

2.1. Cell culture and incubation parameters

Cerebellar granule cells were cultured from the cerebella of 8-day-old rat pups according to Schousboe et al. (1989). Cells were seeded in poly-D-lysine-coated 24-well plastic trays or in plastic dishes (9 cm in diameter), and kept at 37 °C in an atmosphere of air:CO_2, 95:5. On day 2 cytosine D-arabinoside was added to a final concentration of 10 μmol/L to inhibit astrocytic growth. The cultures were used for experiments on day 8 *in vitro*.

For measurements of intracellular calcium and sodium, cerebellar granule cells were cultured on glass coverslips as described (Ring and Tansø, 2007; see below).

In experiments, the culture media were removed and replaced by artificial cerebrospinal fluid (aCSF) containing (in mmol/L) NaCl 94, KCl 25, $NaHCO_3$ 25, NaH_2PO_4 1.4, $CaCl_2$ 1.2, $MgCl_2$ 1.2, giving a total [Na^+] of 120.4. The high [K^+] promotes neuronal survival, and [Na^+] has to be reduced to achieve *iso*-osmolarity (Gallo et al., 1987; Schousboe et al., 1989; Van der Valk et al., 1991). However, in some experiments NaCl was 115 mmol/L (which together with $NaHCO_3$ and NaH_2PO_4 gave a total [Na^+] of 141.4 mmol/L), and KCl was 4 mmol/L; the other salts and glucose were kept constant.

Glucose was 1 mmol/L, because this is close to concentrations that have been measured extracellularly in the brain of wake rats (Kealy et al., 2013). Glucose was increased to 10 mmol/L in some experiments.

NaCl was added to give an additional concentration of 10, 30, 50, 60, or 100 mmol/L. In some experiments excess NaCl (50 mmol/L) was replaced by Na-gluconate or choline-Cl, 50 mmol/L, or mannitol or glycerol, 100 mmol/L. Incubations were done at 37 °C. In some experiments cells were incubated for 20 h with excess NaCl in the absence or presence of MK-801, 5 μmol/L (RBI, Natick, MA, USA) or 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) 10 μmol/L (Tocris Bioscience, Bristol, UK). The solutions were equilibrated with O_2:CO_2 (95:5), and pH was adjusted to 7.3. Experimental incubations took place at 37 °C in an atmosphere of air and CO_2 (95:5).

2.2. Measurements of cell death

Cell death was assessed by the uptake of propidium iodide. Cell death was also assessed with trypan blue uptake (Supplemental Fig. 1), but results were similar to those obtained with propidium iodide; therefore only the latter are reported in the main text. More than 300 cells were counted per culture dish in the propidium uptake studies; more than 200 were counted in the trypan uptake studies. In some experiments cell death was measured as release of lactate dehydrogenase (LDH) to the extracellular fluid as described (Dreiem et al., 2005 and Supplemental Fig. 2). Previously, we have found excellent agreement between dye exclusion tests and LDH measurements in determination of cell death in cultured cerebellar granule cells (Olsen et al., 1999).

2.3. ATP measurements

For ATP measurements cerebellar granule cells were cultured in plastic dishes, 9 cm in diameter. Cells were exposed to aCSF, aCSF + NaCl, 50 mmol/L, or aCSF + mannitol, 100 mmol/L, for 3, 5, or 10 h. At the end of incubation, the media were removed, and the cultures were immersed in liquid N_2. One mL ice-cold perchloric acid, 7% (vol/vol), was sprayed onto the deep frozen cultures, which were then re-immersed in liquid N_2. Cells were scraped off together with the frozen perchloric acid, which was allowed to melt into slurry, transferred to a plastic tube, and centrifuged at 5000*g* to remove protein. The supernatants were brought to pH 8.1 with KOH, 9 mol/L. ATP in the extracts was analyzed fluorometrically according to Lowry and Passonneau (1972). Protein was analyzed according to Lowry et al. (1951).

2.4. Radiolabeling from [^{14}C]glucose. Measurement of amino acids, glucose, and lactate

For metabolic radiotracer studies the culture media were removed, and the cells were washed once with aCSF. Then cultures were incubated for 1 or 10 h with aCSF containing additional NaCl, 50 mmol/L, or mannitol, 100 mmol/L. Glucose was 1 mmol/L. Fifteen minutes before harvesting the cells, 1 μCi of [U-^{14}C]glucose, 3 μCi/μmol (American Radiolabeled Chemicals, St. Louis, MO, USA), was added to the incubation media. At the end of experiments the aCSF was removed, and cultures were washed once with 10 mL ice-cold aCSF before cells were harvested in 200 μL ice-cold perchloric acid, 3% (vol/vol), containing α-aminoadipate, 50 μmol/L, as an internal concentration standard. Protein was removed by centrifugation, and supernatants were neutralized with KOH, 9 mol/L. The precipitating $KClO_4$ was removed by centrifugation, and supernatants were lyophilized to dryness and redissolved in 60 μL double-distilled H_2O. The total levels of amino acids were quantified by HPLC and fluorescence detection after pre-column derivatization with *o*-phthaldialdehyde, as described (Hassel et al., 1995). Radiolabeling of glutamate and aspartate was determined after separation by HPLC as above. The HPLC eluate was collected in 1-minute fractions, and radiolabeling was measured by scintillation counting (Hassel et al., 1995).

Amino acid concentrations in aCSF were determined by HPLC and fluorescence detection as above after mixing the media 1:1 with α-aminoadipate, 50 μmol/L. Concentrations of glucose and lactate were measured by reflectance spectrophotometry with a DT 60 II Ektachem (Kodak, Rochester, NY, USA).

2.5. High-affinity uptake of ^{3}H-labeled glutamate

Cerebellar granule cells were washed once with aCSF and exposed to aCSF containing [2,3-^{3}H]glutamate (24Ci/mmol; final concentration 50 nmol/L; New England Nuclear, Boston, MA, USA) and increasing concentrations of sodium chloride (0–60 mmol/L excess) at room temperature. At 3 min the cultures were washed twice with ice-cold aCSF containing choline chloride instead of NaCl. Blank values were obtained by exposing cells to [2,3-^{3}H] glutamate in sucrose. Blank values were <10% of sodium-dependent values. Cells were harvested in 0.5 mL 70% ethanol, protein was removed by centrifugation, and radioactivity was measured by scintillation counting.

2.6. Measurements of intracellular free [Ca^{2+}] and [Na^+]

Intracellular free [Ca^{2+}] was measured with fura-2/AM (Molecular Probes, Leiden, The Netherlands). Primary cultures of cerebellar granule cells were cultured on 60 × 24 mm rectangular cover slips (Menzel, Germany). Cells were incubated at 37 °C in a