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Short communication

Brain tissue stiffness is a sensitive marker for acidosis

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HIGHLIGHTS

- We introduce tissue stiffness as a sensitive indicator for pathophysiological changes to CNS tissue.
- We applied atomic force microscopy to investigate tissue stiffness.
- We found that CO_2 overexposure-induced acidosis changes brain properties.

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ABSTRACT

Background: Carbon dioxide overdose is frequently used to cull rodents for tissue harvesting. However, this treatment may lead to respiratory acidosis, which potentially could change the properties of the investigated tissue.
New method: Mechanical tissue properties often change in pathological conditions and may thus offer a sensitive generic readout for changes in biological tissues with clinical relevance. In this study, we performed force-indentation measurements with an atomic force microscope on acute cerebellar slices from adult rats to test if brain tissue undergoes changes following overexposure to CO_2 compared to other methods of euthanasia.
Results: The pH significantly decreased in brain tissue of animals exposed to CO_2. Concomitant with the drop in pH, cerebellar grey matter significantly stiffened. Tissue stiffening was reproduced by incubation of acute cerebellar slices in acidic medium.
Comparison with existing methods: Tissue stiffness provides an early, generic indicator for pathophysiological changes in the CNS. Atomic force microscopy offers unprecedented high spatial resolution to detect such changes.
Conclusions: Our results indicate that the stiffness particularly of grey matter strongly correlates with changes of the pH in the cerebellum. Furthermore, the method of tissue harvesting and preparation may not only change tissue stiffness but very likely also other physiologically relevant parameters, highlighting the importance of appropriate sample preparation.
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1. Introduction

Brain tissue is very sensitive to environmental changes, and particularly to a lack of oxygen. CO_2 overdose, which is a standard method to cull rodents for tissue harvesting, leads to an impairment of the oxygen-carbon dioxide exchange, causing death by asphyxiation. It furthermore overloads the capacity of the physiological pH bicarbonate buffering system within the body. This exhaustion of the bicarbonate buffer leads to a respiratory acidosis, *i.e.*, a drop of blood pH below the physiological range of $\sim 7.4 \pm 0.05$ in mammals.

Brain tissue is protected from acidosis by the cerebrospinal fluid, which *in vivo* has a larger buffer capacity than blood (Kazemi et al., 1967). Nevertheless, while the effect of metabolic acidosis (*e.g.*, after ischemia) on brain tissue has been studied in some detail (Kraut and Madias, 2010), it is still largely unclear how the low blood pH after terminal CO_2 overdose affects brain tissue har-

Abbreviations: AFM, atomic force microscopy.

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vested for experimental investigations. Experimental data on pure hypercapnia, obtained by ventilating animals with gas mixtures containing a high CO_2 concentration at normoxia, have shown that brain intracellular pH may drop from a normal value of 7.04 to around 6.65 without inducing permanent damage (Siesjö et al., 1972).

Mechanical tissue properties offer a sensitive readout for chemical changes in biological tissues. Changes in tissue mechanics accompany pathological changes in different organ systems, and they can often be detected even before histological changes are visible. After the induction of liver fibrosis, for example, an increase in liver stiffness precedes the onset of classical fibrosis markers (Georges et al., 2007). Furthermore, in neurodegenerative diseases, such as Multiple Sclerosis (Streitberger et al., 2012) and Alzheimer's disease (Murphy et al., 2011), brain tissue becomes significantly softer. In other organ systems, disease-related changes in tissue stiffness are already used in clinical diagnostics. Mechanical changes in epithelial tissues, for example, are exploited to diagnose breast cancer (Itoh et al., 2006), and an increased arterial stiffness indicates a larger risk for cardiovascular diseases (Mitchell et al., 2010).

Thus, if respiratory acidosis leads to changes in brain tissue architecture and/or function, it is likely that these changes are accompanied by alterations in its mechanical properties. Atomic force microscopy (AFM) is well suited to detect such changes, and its high spatial resolution allows investigating specific regions in the tissue, such as white and grey matter, and even different layers within the grey matter (Fig. 1a).

During an experiment, a small leaf spring – the cantilever – is moved towards the sample until it exerts a set force. This results in the deflection of the cantilever and the indentation of the sample. Cantilever deflection is detected by a laser beam that is reflected off the cantilever's surface onto a photodiode (Fig. 1b). After calibration, this signal is converted into a force. The AFM then records a force-distance curve, plotting the force the cantilever exerts on the sample versus its position relative to the surface (Fig. 1). The apparent elastic modulus of the sample, which is a measure of its elastic stiffness, can be calculated from force-distance curves using the Hertz model. Combining AFM with a motorized microscope stage allows tissue elasticity scans of large areas, and recording forces in the piconewton to nanonewton range with micrometer resolution.

To establish whether respiratory acidosis induced by CO_2 overdose leads to changes in brain tissue, which might impact consecutive measurements of experimental parameters, we induced pH changes in the tissue in different ways and correlated these changes with changes in the tissue's mechanical properties.

2. Materials and methods

2.1. Slice preparation

All procedures were performed according to the UK Animals (Scientific Procedures) Act of 1986. Animals were culled by decapitation, overdose with carbon dioxide or overdose with anesthetic (Pentobarbitone Sodium 20%w/v, Pentoject, LD50 in rats: 118 mg/kg). A power analysis based on previous measurements (Christ et al., 2010) suggested the minimum number of animals required per group to be 3.

For AFM measurements, the brain was removed and kept in ice-cold slicing solution containing 120 mM NaCl, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 2.5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose and 1 mM kynurenic acid (a broad spectrum inhibitor of glutamate receptors used to reduce excitotoxicity), which was oxygenated with 5% CO_2 and 95% O_2, at pH 7,4. Immediately after brain removal, 300 μm slices were cut with a vibratome (VT1200S, Leica, Milton Keynes, UK). Slices were then transferred to a HEPES Ringer solution at pH 7.4, containing 144 mM NaCl, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1 mM NaH_2PO_4, 10 mM HEPES and 10 mM glucose. The buffer was pH adjusted and oxygenated with 100% O_2. Slices were incubated in the HEPES Ringer solution at room temperature for an hour to allow the tissue to equilibrate.

A subset of slices was incubated in oxygenated slicing medium containing amiloride (100 mM) for 10 min and then transferred to a phosphate buffer at pH 6 containing 144 mM NaCl, 2.5 mM KCl, 2.5 mM $CaCl_2$, 8.8 mM NaH_2PO_4, 1.2 mM Na_2HPO_4, 10 mM glucose and optionally 100 mM amiloride. The phosphate buffer was oxygenated with 100% O_2. Slices were attached to plastic culture dishes (TRP, Helene labs) with BD Cell-Tak Cell and Tissue Adhesive (BD Biosciences, Franklin Lakes, USA) and held down with a harp slice grid. For each condition, we used acute slices from at least three animals.

2.2. pH measurements

Immediately after death, skulls were opened and a 3 mm diameter pH probe (InLab®Micro, Mettler Toledo AG, Switzerland) inserted into the cortex. The pH meter readout was recorded every 5 s for the duration of 5 min. The pH probe was calibrated immediately before each measurement and calibration was confirmed after the measurement to exclude drift.

2.3. Atomic force microscopy

37.5 μm polystyrene beads (microParticles GmbH, Berlin, Germany) were glued to tipless cantilevers (k = 0.03–0.05 N/m, Arrow-TL1, NanoWorld, Neuchatel, Switzerland) using UV curable superglue (Loctite, USA). The spring constant k of the cantilevers was measured before attaching the bead using the thermal noise method implemented in the AFM software (JPK Instruments AG, Berlin, Germany).

Force-distance curves were recorded with a Nanowizard III atomic force microscope (JPK Instruments AG, Berlin, Germany) under flux of oxygenated buffer at room temperature similar to electrophysiological set-ups for patch clamp experiments. Slices were indented at 0.3 Hz with a maximum force of 20 pN. The resulting force-distance curves were analyzed using the Hertz model, where

$$F = \frac{4}{3}\frac{E}{(1-\nu^2)}\sqrt{R}\delta^{3/2}$$

Here, F is the applied force, E is the Young's modulus, ν is the Poisson's ratio, R the radius of the probe, and δ the indentation of the sample. Approach curves were analyzed for an indentation of 3 μm as described previously (Christ et al., 2010). The reduced apparent elastic modulus $K = E/(1-\nu^2)$ provides a measure of elastic stiffness: the larger K the stiffer a tissue (Fig. 1c). Values shown correspond to the averages of median values of tissues from individual animals ± standard deviation. By rastering over acute cerebellar tissue slices kept in oxygenated medium, hundreds of force measurements were obtained on each sample.

The averages of the median values of each animal were calculated and significance tested using One Way or Two Way ANOVA tests, followed by a post-hoc Tukey test (OriginPro 8.5, OriginLab Corporation, Northhampton, USA).

3. Results

Approved methods of euthanasia of laboratory rodents include CO_2 overexposure, decapitation, and anesthetic overdose. Since overexposure of CO_2 – but not the other methods – leads to a respiratory acidosis and hence increases blood acidity, we first tested

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