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Original article

Post-sampling release of free fatty acids — effects of heat stabilization and methods of euthanasia

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ABSTRACT

Introduction: The field of lipid research has made progress and it is now possible to study the lipidome of cells and organelles. A basic requirement of a successful lipid study is adequate pre-analytical sample handling, as some lipids can be unstable and postmortem changes can cause substantial accumulation of free fatty acids (FFAs). **Methods:** The aim of the present study was to investigate the effects of conductive heat stabilization and euthanasia methods on FFA levels in the rat brain and liver using liquid chromatography tandem mass spectrometry. **Results:** The analysis of brain homogenates clearly demonstrated phospholipase activity and time-dependent post-sampling changes in the lipid pool of snap frozen non-stabilized tissue. There was a significant increase in FFAs already at 2 min, which continued over time. Heat stabilization was shown to be an efficient method to reduce phospholipase activity and *ex vivo* lipolysis. Post-sampling effects due to tissue thawing and sample preparation induced a massive release of FFAs (up to 3700%) from non-stabilized liver and brain tissues compared to heat stabilized tissue. Furthermore, the choice of euthanasia method significantly influenced the levels of FFAs in the brain. The FFAs were decreased by 15–44% in the group of animals euthanized by pentobarbital injection compared with CO_2 inhalation or decapitation. **Discussion:** Our results highlight the importance of considering euthanasia methods and pre-analytical treatment in lipid analysis, factors which may otherwise interfere with the outcome of the experiments.

1. Introduction

Lipids have highly diverse functions that go beyond their importance for cellular membrane structure and energy storage. These include roles in membrane dynamics, signaling, and energy metabolism (Hannun & Obeid, 2008; Wymann & Schneiter, 2008). The cellular lipidome has been estimated to include about 180 000–200 000 different lipid species (Seppanen-Laakso & Oresic, 2009; Yetukuri, Ekroos, Vidal-Puig, & Oresic, 2008). The most common lipid classes that play important functions in membrane structure and energy storage include glycerophospholipids, triglycerides, sphingolipids, and sterols. Glycerophospholipids and triglycerides consist of fatty acids linked by ester bonds to glycerol, while sphingolipids are sphingoid bases linked by amide bonds to fatty acids. Lipids and deficits in lipid metabolism have been implicated in the pathogenesis of diseases such as insulin-resistant diabetes, Alzheimer's disease, cancer, atherosclerosis and obesity (Loizides-Mangold, 2013). Fatty acids are one class of lipids that are essential components of mammalian cells. They provide energy, function as signaling molecules, sustain structural integrity of cellular membranes, and are of particular importance for the nervous system. Fatty acids are abundant in the nervous system, with concentrations second only to adipose tissue (Etschmaier et al., 2011). The fatty acids participate actively in both the development and the maintenance of the nervous system (Rombaldi Bernardi, de Souza Escobar, Ferreira, & Pelufo Silveira, 2012; Uauy & Dangour, 2006). In addition, free fatty acids (FFAs) have been reported to be involved in pathological conditions of the nervous system, including neurodegenerative diseases, mental disorders and stroke (Hussain, Schmitt, Loeffler, & de Aguilar, 2013). Comprehensive analysis of lipid metabolism is therefore crucial for the understanding of disease mechanisms, and to identify novel disease biomarkers.

With the current advance in mass spectrometry it is now possible to study the lipidome of cells or organelles (Andreyev et al., 2010; Angelini et al., 2012). However, a basic requirement of a successful lipid study is adequate pre-analytical sample handling as some lipids are unstable and postmortem changes in the brain could for instance cause substantial accumulation of FFAs (Bazan, 1970; Birkle & Bazan, 1988; Cenedella, Galli, & Paoletti, 1975). Rapid post-mortem changes of brain constituents have been recognized as a problem for decades, and methods like head-focused microwave irradiation and freeze-blowing have been used in studies to halt enzymatic alterations of the lipid pool in the brain of small experimental animals (Murphy, 2010). Cerebral perfusion

Abbreviations: FFA, free fatty acid; PLA_2, phospholipase A_2.

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of phospholipase inhibitors has also been used for rapid fixation of labile lipid pools in brain tissue (Birkle & Bazan, 1988; Pilitsis, Diaz, O'Regan, & Phillis, 2002). However, most of the studies on brain lipids do not use adequate methods for sample fixation and there is a need for alternative methods that could stabilize various tissues including human samples. Conductive heat stabilization of tissues is an easy and controlled method of sample fixation, which has been shown to stop postmortem protein degradation and counteract post-sampling variation in the proteome (Skold, Alm, & Scholz, 2013; Stingl, Soderquist, Karlsson, Boren, & Luider, 2014; Svensson et al., 2009). The application of oxygen-18 labeling revealed high proteolytic activity in brain samples despite 2 M urea and that heat stabilization of the tissue reduced the protease activity by 97% (Stingl et al., 2014). It is also important to minimize the impact of euthanasia procedures on experimental outcomes. The method of euthanasia may affect the lipidome as the different methods have different mechanisms of action and may induce various level of stress in the animals, which possibly could affect the results as stress may induce lipolysis. In addition, lipid metabolism is influenced by hormones and neuropeptides (Bhathena, 2000). The aim of the present study was to investigate the effects of conductive heat stabilization on FFA levels in the brain of rats, and to compare the effects of different euthanasia methods (i.e. decapitation, CO_2 inhalation, and pentobarbital injection) on FFAs in the brain. The results show that the choice of euthanasia method significantly influences the levels of FFAs in brain tissue, and that heat stabilization is an efficient method to inactivate lipases and reduce post-sampling lipolysis in liver and brain tissue.

2. Materials and methods

2.1. Animals and housing

Wistar rats were obtained from Taconic (Ejby, Denmark) and housed in makrolon cages (59 × 38 × 20 cm) containing wood-chip bedding and nesting material. The animals were maintained on standard pellet food (R36 Labfor; Lantmännen, Kimstad, Sweden) and water *ad libitum*, and were housed in a temperature- and humidity-controlled environment with a 12-h light/dark cycle (lights on at 6 a.m.). The Uppsala animal ethical committee approved all animal experiments, which were performed in accordance with the guidelines of the Swedish legislation on animal experimentation (Animal Welfare Act SFS1998:56) and European Union legislation (Convention ETS123 and Directive 86/609/EEC).

2.2. Postmortem interval effects on FFAs in brain tissue

Twelve rats were killed by decapitation. The brains were removed within 25 s and the cerebellum was subsequently treated using four different protocols (n = 3 per protocol). One group was instantly dissected after rapid enzyme inactivation by heat stabilization of the intact brain, as previously described (Svensson et al., 2009). In short, heat stabilization was performed using the Structure Preserve mode on the Stabilizor T1 (Denator AB, Sweden), which utilizes a combination of heat and controlled pressure to stop degradation while maintaining structural references. During the stabilization, conductive heating plates at 95 °C inactivate enzymes in the sample by a quick and strictly controlled increment of the temperature, resulting in rapid and uniform protein denaturation. Heating times are automatically calculated from the size of each sample (Svensson et al., 2009). The brains from the second and third groups of animals were kept at room temperature for 10 and 20 min, respectively, to allow for post-mortem changes to appear before the heat stabilization of the tissue. After heat stabilization, the samples from all three groups were immediately snap frozen on dry ice. To investigate if heat stabilization of the tissue inhibits postmortem release of FFAs, the brains from the fourth group were instantly dissected after heat stabilization of the brain and left at room temperature for 20 min, before the samples were snap frozen. All samples were stored at −80 °C until FFA analysis.

2.3. Effects of heat stabilization of liver and brain tissue in FFA analysis

To study release of FFAs during sample preparation, six rats were killed by decapitation, the brain and liver were immediately removed, and half of the brain and liver were rapidly heat stabilized whereas tissues from the other half of the tissues were snap frozen on dry ice (n = 6). The samples were stored at −80 °C until FFA analysis.

2.4. Effects of heat stabilization of liver and brain tissues on phospholipase A_2 activity

Six rats were killed by decapitation, the brain and liver were immediately removed, and tissues from three rats were immediately heat stabilized as above, whereas tissues from the other three rats were snap frozen on dry ice (n = 3). All samples were stored at −80 °C until further analysis. Duplicates of all samples were gently homogenized on ice in PBS (100 mg tissue/mL) using a hand-driven glass homogenizer. The phospholipase A_2 (PLA_2) activity was measured for 180 min at room temperature (21 °C) using the fluorometric EnzCheck PLA_2 Assay kit (Invitrogen, Paisley, UK). The samples were analyzed according to the provided standard assay protocol using a Polarstar Optima microplate reader (Bmg Labtech, Offenburg, Germany). To establish that PLA_2 activity corresponded with changes in lipid profile, the time-dependent release of FFAs during the initial linear phase (0–40 min) was analyzed in a brain homogenate. The brain from one rat, killed by decapitation, was immediately removed, snap frozen on dry ice, and stored at −80 °C. Brain tissue (600 mg) was gently homogenized on ice in 6 mL PBS using a hand-driven glass homogenizer, and divided into 12 tubes (0.5 mL per tube). A mix of internal standards ([$^2H_2$] 16:0, [$^{13}C_{16}$]16:1n-7, [$^2H_2$]18:0, [$^2H_2$]18:1n-9, [$^2H_6$]20:3n-6, [$^2H_8$] 20:4n-6, 15:0, 17:0) was added to the tubes and 2 samples were immediately precipitated with 2 mL methanol (0 min). The other samples were left at room temperature (21 °C) and precipitated in duplicates at: 2 min, 5 min, 10 min, 20 min, and 40 min. Fatty acids were subsequently extracted with 4 mL chloroform using a modified Folch procedure and quantified by mass spectrometry, as described below.

2.5. Effects of different euthanasia methods on FFAs in the brain

To compare the effects of euthanasia methods on FFAs in the brain, 24 rats were killed using three different protocols (n = 8 per protocol). The animals in the first group were euthanized by decapitation, using a guillotine. The rats in the second and third group were euthanized by CO_2 inhalation (2 min 30 s, fixed time) and an overdose of pentobarbital (120 mg/kg ip injection), respectively. The brains were immediately removed and the cerebellum was dissected after heat stabilization of the intact brain. The samples were then snap frozen on dry ice and stored at −80 °C until FFA analysis.

2.6. Lipid extraction

Lipids were extracted from brain and liver tissue according to a modified Folch extraction previously described (Astarita, Ahmed, & Piomelli, 2009). In short, 200 mg of rat tissue was gently homogenized on ice in 2 mL ice-cold methanol containing internal standards ([$^2H_2$] 16:0, [$^{13}C_{16}$]16:1n-7, [$^2H_2$]18:0, [$^2H_2$]18:1n-9, [$^2H_6$]20:3n-6, [$^2H_8$] 20:4n-6, 15:0, 17:0) using a hand-driven glass homogenizer. Four mL ice-cold chloroform was added, and the samples were vortexed for 10 s. Water (1.5 mL) was added, followed by 10 s of vortexing. The samples were centrifuged at 1000 × *g* for 20 min at 4 °C, and the bottom lipid phases were transferred to new glass tubes. The upper aqueous phase was re-extracted with 4 mL chloroform, and the centrifugation was repeated. The lipid phases were combined, evaporated under a stream of N_2, and re-constituted in 1 mL methanol. A calibration curve was created using a series of standard mixtures containing FFA. The