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Changes in the hippocampal and peripheral phospholipid profiles are associated with neurodegeneration hallmarks in a long-term global cerebral ischemia model: Attenuation by Linalool

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ABSTRACT

Phospholipid alterations in the brain are associated with progressive neurodegeneration and cognitive impairment after acute and chronic injuries. Various types of treatments have been evaluated for their abilities to block the progression of the impairment, but effective treatments targeting long-term post-stroke alterations are not available. In this study, we analyzed changes in the central and peripheral phospholipid profiles in ischemic rats and determined whether a protective monoterpene, Linalool, could modify them. We used an *in vitro* model of glutamate (125 μM) excitotoxicity and an *in vivo* global ischemia model in Wistar rats. Linalool (0.1 μM) protected neurons and astrocytes by reducing LDH release and restoring ATP levels. Linalool was administered orally at a dose of 25 mg/kg every 24 h for a month, behavioral tests were performed, and a lipidomic analysis was conducted using mass spectrometry. Animals treated with Linalool displayed faster neurological recovery than untreated ischemic animals, accompanied by better motor and cognitive performances. These results were confirmed by the significant reduction in astrogliosis, microgliosis and COX-2 marker, involving a decrease of 24:0 free fatty acid in the hippocampus. The altered profiles of phospholipids composed of mono and poly-unsaturated fatty acids (PC 36:1; 42:1 (24:0/18:1)/LPC 22:6)/LPE 22:6) in the ischemic hippocampus and the upregulation of PI 36:2 and other LCFA (long chain fatty acids) in the serum of ischemic rats were prevented by the monoterpene. Based on these data, alterations in the central and peripheral phospholipid profiles after long-term was attenuated by oral Linalool, promoting a phospholipid homeostasis, related to the recovery of brain function.

1. Introduction

Cerebral ischemia is the third leading cause of death and the first cause of acquired disability worldwide (Bousser, 2012). Ischemia causes significant sensorimotor and cognitive impairments, as well as physical and mental disabilities and other complications, such as post-stroke dementia, depression, falls, fractures, and epilepsy, altering the quality of life of patients and their families (Rothwell et al., 2011). Ischemic stroke is caused by the occlusion of cerebral blood vessels, which deprive brain cells of the oxygen and glucose required for their function. The resulting energy deficiency perturbs mitochondrial ATP synthesis and increases the generation of free radicals, leading to oxidative stress and lipid peroxidation. Concomitantly, the activation of excitatory neurotransmitters such as dopamine and glutamate induces intracellular calcium overload, metabolic dysfunction and acidosis (Moskowitz et al., 2010). An increase in the intracellular calcium concentration activates sphingomyelinases and phospholipases A2, C and D which, in turn, promote the release of second messengers,

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such as diacylglycerol (DAG), phosphatidic acid and arachidonic acid (ArAc), which are involved in inflammation, excitotoxicity and other cell death pathways (Phillis and O'Regan, 2004; Tian et al., 2009).

In general, lipids are complex, diverse and their functions rely on their cellular localization. Lipids are involved in maintaining the membrane structure and may act as metabolic signaling molecules and neuromodulators in the central nervous system (CNS) (Martinez-Gardeazabal et al., 2017). Specifically, phospholipids are critical components of the endolysosomal system and modulate the trafficking of neural cells and the release of neurotransmitters; in patients with a brain disease, modifications to the fatty acid composition of phospholipids affect their homeostasis and function. For example, many phenomena observed during brain ischemia and reperfusion are accounted for by damage to membrane lipids, specifically lipolysis during ischemia and radical-mediated peroxidation of polyunsaturated fatty acids (PUFAs) during reperfusion (Rabiei et al., 2012). However, the role of phospholipids in the long-term post-stroke neuropathogenesis and mechanisms to prevent this disorder are not clearly understood.

The development of new strategies for the prevention and treatment of stroke has grown considerably over the last few years. The main focus of the therapy relies on decreasing the acute disability of stroke through the use of thrombolytic drugs (e.g., recombinant tissue plasminogen activator) to limit the infarct size and improve the outcome, but has the difficulty of only being able to be administered in a small therapeutic window (Schulz, 2013). Currently, several natural products have been used for neuroprotective purposes. Among them, Linalool exhibits different biological properties that could be used to treat cerebrovascular diseases.

Linalool (Lin, 3,7-dimethyl-1,6-octadien-3-ol) is a monoterpene alcohol that is naturally found in some plants (e.g., anise, pepper and fennel), is useful for attracting pollinators in the natural environment (Raguso, 2016), and has been used in the cosmetic and medical industries as a flavoring. Linalool has numerous biological activities, including anti-neoplastic (Miyashita and Sadzuka, 2013), anti-inflammatory (Li et al., 2016), anti-leishmanicidal, antioxidant and antimicrobial activities (Park et al., 2012; Shi et al., 2016). Linalool was recently shown to exert effects on the CNS, including sedative, anti-nociceptive, anticonvulsant, anti-depressive and anxiolytic effects (Coelho et al., 2013; Guzmán-Gutiérrez et al., 2015). It has also been reported to modulate neurotransmission *in vitro* and *in vivo* models through its effect on the NMDA receptor (Elisabetsky et al., 1995). As shown in our recent study, Linalool also exerts neuroprotective effects on a triple transgenic AD mouse model by reversing the histopathological hallmarks and cognitive and emotional dysfunction (Sabogal-Guáqueta et al., 2016). Moreover, in a 2014 study by Mehri et al. of a rat model of acrylamide-induced neurotoxicity, this molecule reduced lipid peroxidation in the brain tissue (Mehri et al., 2015).

In addition, the two-vessel occlusion (2-VO) animal model gives rise to ischemic cell change in a number of brain areas including the hippocampal CA1 subfield, striatum and neocortex (McBean and Kelly, 1998). Although, the infarct volume is not quantifiable in this model, because of the diffuse and variability pattern of the infarcted areas. 2-VO model reproduces neurological, motor and cognitive impairments at short and long-term post-ischemia in agreement with our previous studies (Becerra-calixto and Cardona-gómez, 2016; Gutiérrez Vargas et al., 2010; Villamil Ortiz and Cardona Gomez, 2015).

Therefore, considering the limited state of the art of monitoring phospholipids after stroke, in the present study, we used *in vitro* glutamate excitotoxicity model and *in vivo* 2-VO cerebral ischemia model to context the neural cell imbalance and to understand the affection on the hippocampal and peripheral phospholipid profile, also analyzing whether a Linalool treatment might modify these parameters.

2. Materials and methods

2.1. Animal procedures

All animal procedures were performed in accordance with the ARRIVE guidelines, the Guide for the Care and Use of Laboratory Animals, 8th edition, published by the National Institutes of Health (NIH) and Colombian standards (law 84/1989 and resolution 8430/1993). These procedures were approved by the Ethics Committee for Animal Experimentation of the University of Antioquia, Medellin, Colombia.

2.2. Dissociated cortical neuron and astrocyte cultures

Cerebral cortices and hippocampi from Wistar rat embryos (E18) were dissected, trypsinized and cultured on multi-well plates pre-coated with poly-L-lysine (Sigma-Aldrich) in Neurobasal medium (GIBCO) containing B-27 supplement (Sigma Aldrich) and a penicillin–streptomycin antibiotic mixture (GIBCO) at 37 °C in a 5% CO_2 humidified atmosphere for a maximum of 7 days (DIV 7) or 19 days (DIV 19) *in vitro* for immature and mature cultures, respectively. Neurons that had been isolated and dissociated as described above were plated at a density of 500 cell/mm^2 (equivalent to 100,000 cells per well) in 24-well plates for immunofluorescence and cytotoxicity assays or at a density of 250 cell/mm^2 (50,000 cells per well) in 96-well plates. On the other hand, the cortices and hippocampi of neonate Wistar rats (PN1–2) were dissected, trypsinized, dissociated, and cultured in 75-cm^2 flasks at 37 °C and 5% CO_2 to obtain astrocytes (Posada-Duque et al., 2015). The cell confluence was observed at DIV10 and was approximately 4×10^6 cells. Subsequently, astrocytes were subcultured at DIV 13–14, by treatment with 0.25% trypsin/EDTA (GIBCO) and replated in 12- or 24 well plates at densities of 7.5×10^4 and 3.5×10^4 cells per well, respectively.

2.2.1. LDH release

Linalool was dissolved in DMSO (Dimethyl sulfoxide) and added to cultures of neurons or astrocytes at different concentrations (0.1 μM, 1 μM, 10 μM, 100 μM and/or 200 μM). Cytotoxicity was assessed by measuring the amount of lactate dehydrogenase (LDH) released from the cultures using a cytotoxicity detection kit (Cat. No. 11644793001-Roche Molecular Biochemicals, Indianapolis, IN, USA) as previously described (Posada-Duque et al., 2013). All experiments were performed three times in duplicate.

2.2.2. In vitro protection assays

Neuronal and astrocytes cultures from cortices and hippocampi were treated with 125 μM glutamate as toxic stimulus and glutamate buffer (pH = 7) as vehicle for 20 min and post-treated with Linalool (n = 3–4) at concentration of 0.1 μM. We evaluated LDH release from mature neurons at DIV 19 and astrocytes at DIV 24, also the percentage of condensed nuclei under the same conditions using the following formula: [condensed nuclei/(condensed nuclei + normal nuclei)] × 100 (Posada-Duque et al., 2013). Analyses were performed for 30 neurons per treatment in each duplicate assay from at least three or four independent experiments.

2.2.3. ATP quantification assay

Homogenized cells were used to quantify ATP levels with the CellTiter-Glo Luminescent Cell Viability Assay (Promega,