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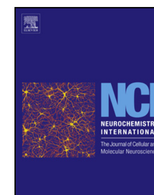

Further evidence for the neuroprotective role of oleanolic acid in a model of focal brain hypoxia in rats

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ABSTRACT

Ischemic brain injury is a dynamic process involving oxidative stress, inflammation, cell death and the activation of endogenous adaptive and regenerative mechanisms depending on the activation of transcription factors such as hypoxia-inducible factor 1-alpha. Accordingly, we have previously described a new focal hypoxia model by direct intracerebral cobalt chloride injection. In turn, oleanolic acid, a plant-derived triterpenoid, has been extensively used in Asian countries for its anti-inflammatory and anti-tumor properties. A variety of novel pharmacological effects have been attributed to this triterpenoid, including beneficial effects on neurodegenerative disorders – including experimental autoimmune encephalomyelitis – due to its immunomodulatory activities at systemic level, as well as within the central nervous system. In this context, we hypothesize that this triterpenoid may be capable of exerting neuroprotective effects in ischemic brain, suppressing glial activities that contribute to neurotoxicity while promoting those that support neuronal survival. In order to test this hypothesis, we used the intraperitoneal administration of oleanoic acid in adult rats for seven days previous to focal cortical hypoxia induced by cobalt chloride brain injection. We analyzed the neuroprotective effect of oleanoic acid from a morphological point of view, focusing on neuronal survival and glial reaction.
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1. Introduction

Traditional medicines and phytopharmaceutical compounds have both been used for the treatment of inflammatory and degenerative diseases. For example, the Mediterranean diet, whose health benefits have long been attributed to a high content of monounsaturated fatty acids and in which olive oil is the major source of dietary fat intake, has been associated with low incidence of cardiovascular diseases (Chiva-Blanch et al., 2014; Delgado-Lista et al., 2014) and cancer (Bao et al., 2014; Villar et al., 2014).

Terpenes in general, and triterpenes in particular, show anti-inflammatory activity and act as immunomodulators in nutraceutical agents. In particular, oleanolic acid (OA) (3b-hydroxy-olea-12-en-28-oic acid), a pentacyclic triterpene, is a natural compound found in various plants, fruits and herbs, and it is isolated from chloroform extract of *Olea ferruginea Royle* after the removal of organic bases and free acids (Sultana and Saify, 2012).

Several studies have been shown promising effects including anti-neoplastic (Srivastava et al., 2010), gastroprotective (Rodriguez et al., 2003), antibacterial (Fontanay et al., 2008) and anti-inflammatory (Lee et al., 2013). Neuroprotective effects were also described for OA in models of degenerative disease. OA suppreses NF-κB p65, Bax and cleaved caspase-3 production, and retains Bcl-2 expression (Tsai and Yin, 2012). In inflammatory demyelinating diseases like multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis (EAE), the OA protected against EAE by restricting infiltration of inflammatory cells into the central nervous system (CNS) and by preventing blood–brain barrier disruption (Martin et al., 2012).

Often a result of ischemic stroke, hypoxia is defined as a situation in which O_2 supply is insufficient for normal metabolism, which produces a reduction in aerobic metabolism, loss of cellular function and eventual cell death (Ratan et al., 2007). With an approximate global incidence of 250–400 in 100,000 and a mortality rate of around 30%, stroke is the third most common cause of mortality and one of the leading causes of long-term disability (Lloyd-Jones et al., 2010). According to the World Health Organization, 15 million people suffer stroke worldwide every year, out of whom 5 million

Abbreviations: $CoCl_2$, cobalt chloride; OA, oleanolic acid; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; SS, saline solution; i.p, intraperitoneal; PB, phosphate buffer; PBS, phosphate-buffered saline; GFAP, glial fibrillary acidic protein; MAP-2, microtubule-associated protein-2; iNOS, inducible nitric oxide synthase; VIM, vimentin; NADPH-D, nicotinamide adenine dinucleotide phosphate diaphorase.

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die and another 5 million are permanently disabled (Mackay and Mensah, 2004). Although there are currently no effective treatments to enhance functional recovery following stroke, which is why it poses a massive socio-economic burden worldwide (Kunz et al., 2010), *in vitro* and *in vivo* models of hypoxia have shown neuroprotective effects against ischemic injury conferred by novel tritepenoid compound (Zhang et al., 2012).

We have previously used a hypoxia model, consisting in an intracortical injection of $CoCl_2$, which is widely used to induce hypoxic conditions both *in vivo* and *in vitro* (Karovic et al., 2007). Cobalt inactivates HIF-specific proline-hydroxylases (Berra et al., 2006; Epstein et al. 2001), impairing the binding of von Hippel–Lindau protein with HIF-1α (Yuan et al., 2003), causing the stabilization of HIF-1α and preventing its degradation by the proteasome. This in turn produces a focal hypoxia-like lesion by stabilizing and inducing HIF-1a and exhibiting neuronal and glial alterations (Caltana et al., 2009).

On the basis of the apparent protective effects of the Mediterranean diet against cardiovascular diseases, its high concentration of triterpenes such as oleanolic acid, and previous reports where the pretreatment with triterpenes protected the heart against myocardial infarction (Janahmadi et al., 2014), the aim of this work was to analyze the potential neuroprotective effect of OA pre-treatment (as a tool to prevent or attenuate the consequences) in a model of focal cerebral chemical hypoxia, focusing on neuronal survival and glial reaction.

2. Materials and methods

2.1. Animal treatment

Animal care for this experimental protocol was in accordance with the NIH guidelines for the Care and Use of Laboratory Animals and the principles presented by the Society for Neuroscience in the Guidelines for the Use of Animals in Neuroscience Research, and authorized by the CICUAL (Comité Institucional de Cuidado y Uso de Animales de Experimentación, School of Medicine, University of Buenos Aires). All efforts were made to reduce suffering and the number of animals used.

Sixteen adult male Wistar rats (250–300 g) obtained from the animal facility in the School of Pharmacy and Biochemistry, University of Buenos Aires, were used in this study. Rats were housed in a controlled environment (12 h/12 h light/dark cycle, controlled humidity and temperature, free access to standard laboratory rat food and water).

First, rats were divided into two groups –OA and SS–, which received, from day 1 to day 7, an intraperitoneal (i.p.) injection of either OA (6 mg/kg/day dissolved in DMSO and saline solution) or vehicle (DMSO dissolved in an equivalent volume of saline solution), respectively. Each group was in turn divided in two subgroups –$CoCl_2$ and SS– which received, on day 8 and at intracortical level, a surgically administered injection of either a solution of 50 mM cobalt chloride ($CoCl_2$) or saline solution (Caltana et al., 2009), respectively. Briefly, animals were subjected to a unilateral lesion by placing them in a stereotaxic apparatus and drilling a small hole on the skull in the frontoparietal cortex at Bregma -1.30 mm (Paxinos and Watson, 2013). Once the underlying pia was reached, a Hamilton syringe was used to inject sterile solution of $CoCl_2$ (50 mM) or saline solution in the right hemisphere, 1 mm below the pia level in the cerebral cortex (layers 3–4). The $CoCl_2$ solution was prepared in sterile-diluted saline solution adjusted to reach a physiological osmolarity of 310 mOsm/kg soon after the $CoCl_2$ was added. Then, the incision in the overlying skin was closed using the temporal muscle and the attached fascia to cover the lesion site. During surgery and the whole emergence period, animals' body temperature was maintained by means of a heating pad. Surgical procedures were performed under anesthesia induced with sevofluorane 8% (v/v) and each animal was later put in a separate cage for recovery. For two more days following surgical procedures, rats continued receiving OA or SS.

Experimental groups were thus as follows:

Control groups:

Group 1: SS-SS: intracerebrally injected with saline and pretreated with vehicle.
Group 2: SS-OA: intracerebrally injected with saline and pretreated with OA.

Hypoxic groups:

Group 3: $CoCl_2$-SS: intracerebrally injected with $CoCl_2$ and pretreated with vehicle.
Group 4: $CoCl_2$-OA: intracerebrally injected with $CoCl_2$ and pretreated with OA.

On the 11th day each animal was deeply anesthetized with sevofluorane 8% (v/v) and perfused through the left ventricle, initially with saline solution added to 50 IU heparin and 0.05% (w/v) $NaNO_2$, and subsequently with a fixative solution containing 4% (w/v) paraformaldehyde and 0.25% (v/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 (PB). Brains were removed and postfixed in the same cold fixative solution for 2 h. Brains were then washed three times in cold 0.1 M phosphate buffer, pH 7.4, containing 5% (w/v) sucrose, and left in the same washing solution for 18 h at 4 °C. Finally, brains were cryoprotected by immersion in a solution containing 25% (w/v) sucrose in PB and stored at −20 °C until used. Coronal 40-μm-thick brain sections corresponding to the area of saline and $CoCl_2$ injection sites were obtained using a cryostat and mounted on gelatin-coated slides.

2.2. Fluoro-Jade® B staining procedure

Brain sections were mounted with distilled water onto gelatin-coated slides and dried on a slide warmer at 50 °C for at least half an hour. Sections were then immersed in a solution containing 1% (w/v) sodium hydroxide in 80% alcohol for 5 minutes, followed by 2 minutes in 70% (v/v) alcohol and 2 minutes in distilled water. The slides were then transferred to a solution of 0.06% (w/v) potassium permanganate for 10 minutes. Slides were rinsed for 2 minutes in distilled water and then transferred to the Fluoro-Jade® B: 0.0004% staining solution for 20 minutes. After staining, sections were rinsed three times in distilled water. Excess water was drained off and slides were rapidly set at approximately 50 °C, until they were fully dry. Once dry, slides were immersed in xylene and then coverslipped using Permount mounting media. Sections were examined with an epifluorescence microscope using a filter system suitable for fluorescein or FITC visualization (Balan et al., 2006).

2.3. Immunohistochemistry

Cryostat brain sections of animals belonging to the different experimental groups were simultaneously processed as previously described (Evrard et al., 2006). Briefly, after phosphate-buffered saline (PBS) rinses, endogenous peroxidase activity was inhibited with 0.5% (v/v) H_2O_2 in PBS for 30 minutes at room temperature. Brain sections were then blocked for 1 hour with 3% (v/v) normal goat serum in PBS. After rinsing in PBS, sections were incubated for 48 hours at 4 °C with the primary antibodies and then rinsed and incubated 1 hour at room temperature with biotinylated secondary antibodies (1:400). After further washing in PBS, sections were incubated for 1 hour with the Extravidin complex solution (1:400).

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