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Time and dose dependent effects of oxidative stress induced by cumene hydroperoxide in neuronal excitability of rat motor cortex neurons

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ABSTRACT

It has been claimed that oxidative stress and the production of reactive oxygen radicals can contribute to neuron degeneration and might be one factor in the development of different neurological diseases. In our study, we have attempted to clarify how oxidative damage induces dose dependent changes in functional membrane properties of neurons by means of whole cell patch clamp techniques in brain slices from young adult rats. Our research demonstrates physiological changes in membrane properties of pyramidal motor cortex neurons exposed to 3 concentrations of cumene hydroperoxide (CH; 1, 10 and 100 μM) during 30 min. Results show that oxidative stress induced by CH evokes important changes, in a concentration and time dependent manner, in the neuronal excitability of motor cortex neurons of the rat: (i) Low concentration of the drug (1 μM) already blocks inward rectifications (sag) and decreases action potential amplitude and gain, a drug concentration which has no effects on other neuronal populations, (ii) 10 μM of CH depresses the excitability of pyramidal motor cortex neurons by decreasing input resistance, amplitude of the action potential, and gain and maximum frequency of the repetitive firing discharge, and (iii) 100 μM completely blocks the capability to produce repetitive discharge of action potentials in all cells. Both larger drug concentrations and/or longer times of exposure to CH narrow the current working range. This happens because of the increase in the rheobase, and the reduction of the cancelation current. The effects caused by oxidative stress, including those produced by the level of lipid peroxidation, are practically irreversible and, this, therefore, indicates that neuroprotective agents should be administered at the first symptoms of alterations to membrane properties. In fact, the pre-treatment with melatonin, acting as an antioxidant, prevented the lipid peroxidation and the physiological changes induced by CH. Larger cells (as estimated by their cell capacitance) were also more susceptible to oxidative stress. Our results provide previously unavailable observations that large size and high sensitivity to oxidative stress (even at low concentrations) make pyramidal neurons of the motor cortex, in particular corticofugal neurons, more susceptible to cell death when compared with other neuronal populations. These results could also shed some light on explaining the causes behind diseases such as Amyotrophic Lateral Sclerosis.

1. Introduction

Oxidative stress is a pathological condition which implies overproduction of Reactive Oxygen Species (ROS) under conditions in which the cell's antioxidant defense system becomes no longer effective (Heather and Teismann, 2009; Sims-Robinson et al., 2013). ROS are generated as a consequence of an aerobic metabolism determined by mitochondrial respiration (Hool, 2006). To eliminate these ROS, cells develop several protective mechanisms. Among them, enzymes such as superoxide dismutases, catalases and glutathione peroxidases directly transform some ROS into compounds of lower toxicity through the oxidation of antioxidant metabolites: reduced glutathione, thioredoxin, and ascorbic acid (Reynolds et al., 2007; Sha et al., 2013). Low level of ROS participate in cell division and growth regulation, apoptosis regulation, oxidative modifications of biomolecules in extracellular space, protection from pathogen invasion, etc. (Han et al., 2008). High levels of ROS may appear in other situations (such as mitochondrial dysfunction, excitotoxic insult, or inflammation), and cause DNA mutations, ion channel damage, intensification of the lipid peroxidation process, and oxidation of proteins and other biomolecules (Lambert et al., 2004; Ljubisavljevic, 2014; Pitt et al.,

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2000) which lead to impairing cell function. They may also affect different transcription factors, growth factors, kinases phosphatases and cytokines (Arrigo, 1999; Emerit et al., 2004; Tirosh et al., 2000; Valencia and Moran, 2004; Vimard et al., 2011). At the level of the membrane, ROS alter ATP-sensitive K^+ currents, L-type Ca^{2+} currents (Goldhaber and Liu, 1994; Racay et al., 1997) and delayed rectifier K^+ currents (Goldhaber et al., 1989), ions transporters (Kourie, 1998), either through direct oxidation of lipids, or through alterations of cell membrane proteins and intracellular signaling pathways (Hool, 2006; Zhu et al., 2005). ROS can also affect membrane properties including variations in cell cytoskeletal architecture and membrane stiffness, membrane potential, ionic gradients, action potential duration and amplitude, spontaneous activity, and excitability (Jovanovic and Jovanovic, 2013; Nakaya et al., 1992; Nani et al., 2010; Pardillo-Díaz et al., 2015; Sinha et al., 2015).

Lipid peroxidation (LPO) is one of the most commonly studied processes of redox cell signalization disorders where free radicals have a great importance (Ayala et al., 2014). Through different mechanisms, LPO disrupts membrane barrier function, inactivates membrane enzymes, and increases permeability for water, monovalent and divalent ions, and even high molecular weight compounds (Ferretti and Bacchetti, 2011; Ljubisavljevic, 2014; Nam, 2011). Cumene hydroperoxide (CH) is a stable organic oxidizing agent that is known to penetrate into the inner hydrophobic part of the membrane lipid bilayer, causing extensive peroxidation of lipids (to a much greater extent than hydrogen peroxide) (Jovanovic and Jovanovic, 2013; van den Berg et al., 1992). CH has also been described to be able to react with aminoacids and proteins with multiple effects, such as oxidation of side-chains, backbone fragmentation, dimerization/aggregation, unfolding or conformational changes, enzymatic inactivation, and alterations in cellular handling and turnover of proteins, as singlet oxygen does (Ayala et al., 2014; Davies, 2003; Gracanin et al., 2009). Consequently, CH has been used to inflict oxidative stress in vitro in neurons (Jovanovic and Jovanovic, 2013; Nakaya et al., 1992; Nani et al., 2010; Pardillo-Díaz et al., 2015). Regarding neuronal cell damage, it is widely believed that oxidative stress has a fundamental role in neuronal degeneration and might be one factor in the development of different diseases, such as Amyotrophic Lateral Sclerosis (ALS), Parkinson's, Schizophrenia, Alzheimer's (Andersen, 2004; Cabungcal et al., 2014; Cleveland and Rothstein, 2001; Reynolds et al., 2007) and ageing (Muller et al., 2007). ALS is a progressive neurodegenerative disease that results from the death of the upper motor neurons of the motor cortex, including layer pyramidal neurons, that regulate voluntary control of motor output (Mochizuki et al., 2011). In vitro and in vivo clinical and preclinical studies show that ALS is characterized by higher levels of oxidative stress biomarkers and by lower levels of antioxidant defense biomarkers in the brain and peripheral tissues (reviewed in Niedzielska et al., 2015). Post-mortem studies on tissue samples from ALS patients support the hypothesis of the oxidative damage in proteins, lipids, and DNA (Bogdanov et al., 2000; Ihara et al., 2005; Smith et al., 1998; Tohgi et al., 1999). Indeed, recent positron emission tomography (PET) imaging data in humans have confirmed that oxidative stress is enhanced in motor cortex in ALS patients compared with controls (Ikawa et al., 2014). Moreover, recent studies have demonstrated extensive early changes to the morphology of motor cortex neurons in SOD1 mice (mouse model of ALS based on oxidative stress), which thus confirms clinically relevant cortical pathophysiology more faithfully than previously thought (Fogarty et al., 2015; Saba et al., 2015). All this evidence leads to the conclusion that oxidative stress may be an important factor associated with the development of neurodegeneration in ALS patients.

In our study, we have attempted to clarify how oxidative stress evokes dose dependent changes in the functional properties of neurons. In previous studies, we have demonstrated that oxidative stress compromises both neuronal excitability and the capability of generating action potentials (Pardillo-Díaz et al., 2015). In order to understand how oxidative stress modifies neuron cell membrane properties, we have used three concentrations of CH at quantities that do not induce early cell death (Shimura et al., 1985; Vimard et al., 1996, 2011; Vroegop et al., 1995). We have registered the electrophysiological properties of pyramidal motor cortex neurons in brain slices by means of whole cell recordings. Our study aims to answer important questions such as: (i) Does acute oxidative stress induce functional changes in a dose dependent manner? (ii) In case such changes were dose dependent, are some membrane properties more sensitive to the drug concentration? (iii) Can membrane properties be recovered to resting values after washout of the drug? (iv) Can the pre-treatment with antioxidant agents, such as melatonin, prevent the LPO and the physiological changes induced by CH? (v) Are alterations in membrane properties dependent of neuronal size? The answers to these questions could be potentially relevant to explain the role oxidative damage may have in neurological disease, such as ALS.

2. Experimental procedures

The method has been described in detail in a previous publication by our lab team (Pardillo-Díaz et al., 2015). Briefly, the present study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the European Community Directive 2003/65 and the Spanish Royal Decree 120/2005. The research protocol was approved by the Animal Ethics Committee of the University of Seville. Wistar rats (20–40 days of age) were deeply anaesthetized with chloral hydrate (4%, Panreac). Brains were quickly removed and placed in ice-cold low-calcium Artificial Cerebro-Spinal Fluid (ACSF). Transverse sections (thickness 300 μm) of the primary motor cortex were cut off on a vibratome (NVLSM1, WPI), placed in an ACSF-filled chamber for 30 min at ~37 °C, and then stored at ~21 °C. The composition of the ACSF (in mM) was as follows: 126 NaCl, 2 KCl, 1.25 Na_2HPO_4, 26 $NaHCO_3$, 10 glucose, 2 $MgCl_2$, and 2$CaCl_2$. For the low-calcium-ACSF solution, the concentrations were 4 $MgCl_2$ mM and 0.1 $CaCl_2$ mM. Both ACSF and low-calcium-ACSF solutions were bubbled with 95% O_2–5% CO_2 (pH 7.4).

2.1. Whole-cell patch-clamp recordings

Slices containing the primary motor cortex were transferred to a recording chamber and superfused at 1–2 ml min^{-1} with circulating aerated ACSF warmed to 33° ± 1 °C via a feedback-controlled heater (TC 324B; Warner). Neurons were patch-clamped under direct visual control using a Nikon Eclipse FN1 microscope equipped with infrared-differential interference contrast (IR-DIC) optics, a 40× water immersion objective, and a WAT-902H2 Ultimate Camera. Cortical pyramidal neurons were distinguished by their typical morphology (Stuart et al., 1993) including a large pyramidal-shaped soma and a prominent apical dendrite extending vertically toward the pial surface. Patch pipettes were pulled (PC-10, Narishige, Tokyo, Japan) from borosilicate glass capillaries with filament (inner diameter 0.6, outer diameter 1 mm; Narishige) to a tip resistance of 3–5 MΩ. Patch pipettes were filled with (in mM): 120 K-gluconate, 10 KCl, 10 phosphocreatine disodium salt, 2 MgATP, 0.3 NaGTP, 0.1 EGTA, 10HEPES, adjusted to pH 7.3 with KOH. The osmolarity of the intracellular solution was 285 mosmol/kg, adjusted with sucrose. Whole-cell recording configuration was obtained using a micromanipulator (MP-225, Sutter) and a patch-clamp amplifier