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OXIDATIVE STRESS INDUCED BY CUMENE HYDROPEROXIDE EVOKES CHANGES IN NEURONAL EXCITABILITY OF RAT MOTOR CORTEX NEURONS

R. PARDILLO-DÍAZ,[a] L. CARRASCAL,[a] A. AYALA[b] AND P. NUNEZ-ABADES[a]*

[a] *Department of Physiology, School of Pharmacy, University of Seville, Spain*

[b] *Department of Biochemistry and Molecular Biology, School of Pharmacy, University of Seville, Spain*

Abstract—Oxidative stress and the production of reactive oxygen radicals play a key role in neuronal cell damage. This paper describes an *in vitro* study that explores the neuronal responses to oxidative stress focusing on changes in neuronal excitability and functional membrane properties. This study was carried out in pyramidal cells of the motor cortex by applying whole-cell patch-clamp techniques on brain slices from young adult rats. Oxygen-derived free radical formation was induced by bath application of 10 μM cumene hydroperoxide (CH) for 30 min. CH produced marked changes in the electrophysiological properties of neurons ($n = 30$). Resting membrane potential became progressively depolarized, as well as depolarization voltage, with no variations in voltage threshold. Membrane resistance showed a biphasic behavior, increasing after 5 min of drug exposure and then it started to decrease, even under control values, after 15 and 30 min. At the same time, changes in membrane resistance produced compensatory variations in the rheobase. The amplitude of the action potentials diminished and the duration increased progressively over time. Some of the neurons under study also lost their ability to discharge action potentials in a repetitive way. Most of the neurons, however, kept their repetitive discharge even though their maximum frequency and gain decreased. Furthermore, cancelation of the repetitive firing discharge took place at intensities that decreased with time of exposure to CH, which resulted in a narrower working range. We can conclude that oxidative stress compromises both neuronal excitability and the capability of generating action potentials, and so this type of neuronal functional failure could precede the neuronal death characteristics of many neurodegenerative diseases.

Key words: amyotrophic lateral sclerosis, cumene hydroperoxide, lipid peroxidation, neuronal excitability, patch clamp, pyramidal neurons.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by the loss of motoneurons. In a small portion of cases, the origin of the disease is believed to have a genetic component with an explanation to be found in mutations in the cytosolic Cu/Zn superoxide dismutase 1 (SOD-1) gene. In the remaining cases, what causes sporadic ALS still remains rather unclear. One possible theory for the pathogenesis of sporadic ALS is based on excitotoxicity that might trigger mitochondrial dysfunction and impair Ca^{2+} homeostasis and protein aggregation. Another widely accepted theory to explain the appearance of ALS points to oxidative stress (Cleveland and Rothstein, 2001). Oxidative stress is caused when the production of reactive oxygen species (ROS) exceeds the antioxidant capacity of tissues. In neuronal cell damage, this stress is believed to greatly contribute to neuronal degeneration and might be one factor in the development of different diseases, including ALS but also others such as Parkinson's and Alzheimer's (Andersen, 2004; Reynolds et al., 2007) and aging (Muller et al., 2007). Nevertheless, the specific mechanisms responsible for neuronal injury and death remain unknown (Andersen, 2004).

ROS in low levels are generated in all cells as a consequence of the aerobic metabolism determined by mitochondrial respiration (Hool, 2006). To eliminate these ROS, cells develop several protective mechanisms. Superoxide dismutases, catalases and glutathione peroxidases directly transform some ROS into compounds of lower toxicity through the oxidation of important antioxidant metabolites: reduced glutathione, thioredoxin, and ascorbic acid (Reynolds et al., 2007; Sha et al., 2013). High levels of ROS appear in many situations (mitochondrial dysfunction, excitotoxic insult, or inflammation), and cause damage to proteins, DNA and lipids, which leads to impaired cell functions. They may affect different transcription factors, growth factors, kinases phosphatases and cytokines (Arrigo, 1999; Tirosh et al., 2000; Emerit et al., 2004; Valencia and Moran, 2004; Vimard et al., 2011). Moreover, 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.,

*Corresponding author. Address: Departamento de Fisiología, Facultad de Farmacia, Universidad de Sevilla, C/ Profesor García González n°2, 41012 Sevilla, Spain. Tel: +34-954556130.
E-mail address: pnunez@us.es (P. Nunez-Abades).

Abbreviations: ACSF, artificial cerebrospinal fluid; ALS, amyotrophic lateral sclerosis; CH, cumene hydroperoxide; EGTA, ethyleneglycol-bis (2-aminoethylether)-N,N,N′,N′-tetra acetic acid; H_2O_2, hydrogen peroxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ROS, reactive oxygen species.

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1989), ions transporters (Kourie, 1998), either through direct oxidation of lipids, or through alterations of cell membrane proteins and intracellular signaling pathways (Zhu et al., 2005; Hool, 2006).

Cell membrane is particularly vulnerable to oxidative stress due to its lipid and protein composition. Experimental studies have shown that some of the possible effects of reactive oxygen metabolites on membrane properties could include variations in membrane potential, ionic gradients, action potential duration and amplitude, spontaneous activity, and excitability (Nakaya et al., 1992; Nani et al., 2010; Jovanovic and Jovanovic, 2013). In our study, we have tried to clarify how oxidative stress evokes changes in functional properties of neurons by using an *in vitro* model of wild-type rat frontal cortex slices that contained the motor cortex. Our main goal has been to examine the salient effects on membrane properties when oxidative stress is induced to the motor cortex, in an attempt to understand the death of motor neurons that characterizes ALS (Kim et al., 2014). The primary motor cortex plays a central role in controlling movement execution through its projection to the spinal cord and it is also involved in the acquisition of novel movement sequences and skills (Hosp et al., 2011; Oswald et al., 2013).

Cumene hydroperoxide (CH) is a stable organic oxidizing agent (C_6H_5–$(CH_3)_2$–COOH) with a peroxy function group, —O—O—. CH produces lipophilic cumoxyl and cumoperoxyl radicals. CH in the presence of transition metal ions produces cumoxyl radicals, which subtract a hydrogen from the lipid molecule, thus generating the initiation or propagation of lipid peroxidation (Ayala et al., 2014). CH can also react with aminoacids and proteins producing 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 (Davies, 2003; Gracanin et al., 2009). In order to understand how oxidative stress modifies neuronal membrane properties, we have used CH in pyramidal neurons of the motor cortex to a concentration of 10 μM, a measure that does not induce early cell death, as demonstrated by Vimard et al. (2011). More specifically, we have thoroughly looked into the electrophysiological properties directly related to neuronal excitability. To reach this goal, we have registered the electrophysiological properties of the pyramidal motor cortex neurons in brain slices by means of whole-cell recordings. Our data indicate that CH depresses the excitability of pyramidal motor cortex neurons decreasing input resistance, the amplitude of the action potential, and even producing a cancelation of the repetitive firing discharge. As a conclusion, the global effect of this oxidative challenge causes a depression in the cell excitability that may be associated with neuronal death.

EXPERIMENTAL PROCEDURES

This 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; $n = 24$) of both sexes were deeply anaesthetized with chloral hydrate (4%, Panreac). Brains were quickly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF). This cutting solution was a low-calcium-ACSF. Transverse sections (thickness 300 μm) that contained 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 in the same solution until use. 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).

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 recirculating aerated ACSF warmed to 33° ± 1 °C via a feedback-controlled heater (TC 324B; Warner). Motoneurons were patch clamped under visual guidance 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 (Feldman and Kastin, 1984) including a rhomboidal cell body, a prominent apical dendrite extending vertically toward the surface and basal dendrites radiating out from the base of the soma. 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, 10 HEPES, 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 (Multiclamp 700B, Axon Instruments, Molecular Devices, Sunnyvale, CA, USA). Giga seals (> 1 GΩ) were always obtained before rupture of the patch and pipette capacitance was compensated before breaking in, and, in current-clamp mode, the bridge was periodically balanced using the auto-adjust feature. Series resistance was 20 MΩ or less during recording. Current-clamp recordings were low-pass Bessel-filtered at 10 kHz; data were digitized at 2–20 kHz with a Digidata 1550 analog-to-digital converter and acquired using the pCLAMP 10 software (Molecular Devices). Data were stored on a computer disk and analyzed offline using the Clampfit 10.4 software (Molecular Devices).

Drugs and general protocol

Cumene hydroperoxide (CH, Sigma–Aldrich, St. Louis, MO, USA) was prepared just prior to experiments from