Neurobiology of Aging 38 (2016) 73–81

Contents lists available at ScienceDirect

Neurobiology of Aging

journal homepage: www.elsevier.com/locate/neuaging

Age-dependent action of reactive oxygen species on transmitter release in mammalian neuromuscular junctions

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ARTICLE INFO

Article history:
Received 16 May 2015
Received in revised form 13 October 2015
Accepted 26 October 2015
Available online 31 October 2015

Keywords:
Acetylcholine release
Neuromuscular
Reactive oxygen species
Newborn
Aging

ABSTRACT

Reactive oxygen species (ROS) are implicated in aging, but the neurobiological mechanisms of ROS action are not fully understood. Using electrophysiological techniques and biochemical assays, we studied the age-dependent effect of hydrogen peroxide (H_2O_2) on acetylcholine release in rat diaphragm neuromuscular junctions. H_2O_2 significantly inhibited both spontaneous (measured as frequency of miniature end-plate potentials) and evoked (amplitude of end-plate potentials) transmitter release in adult rats. The inhibitory effect of H_2O_2 was much stronger in old rats, whereas in newborns tested during the first postnatal week, H_2O_2 did not affect spontaneous release from nerve endings and potentiated end-plate potentials. Proteinkinase C activation or intracellular Ca^{2+} elevation restored redox sensitivity of miniature end-plate potentials in newborns. The resistance of neonates to H_2O_2 inhibition was associated with higher catalase and glutathione peroxidase activities in skeletal muscle. In contrast, the activities of these enzymes were downregulated in old rats. Our data indicate that the vulnerability of transmitter release to oxidative damage strongly correlates with aging and might be used as an early indicator of senescence.

1. Introduction

It has been well documented that in physiological state, reactive oxygen species (ROS) play a key role in physiological cell signaling, but increased level of ROS production (and/or decreased antioxidant and repair systems activity) leading to increased oxidative stress are implicated in the development of different pathologies (Droge, 2002; Rhee, 2006; Sena and Chandel, 2012). The nervous system is highly vulnerable to oxidative stress and redox imbalance, which contributes to neuronal apoptosis and cognitive decline resulting in a number of neurodegenerative disorders including Parkinson's disease (Sanders and Timothy Greenamyre, 2013; Zuo and Motherwell, 2013), Alzheimer's disease (Jomova et al., 2010; Yan et al., 2013), and amyotrophic lateral sclerosis (ALS; D'Amico et al., 2013; Naumenko et al., 2011; Shi et al., 2010). Aging is associated with increased oxidative damage and failure of antioxidant defense, which result in higher incidence of a wide range of the oxidative stress-induced neurodegenerative processes (Balaban et al., 2005; Jackson and McArdle, 2011; Radak et al., 2013; Salminen and Paul, 2014). Nevertheless, there is limited information on the impact of ROS on the function of synapses, key elements of the nervous system. In particular, there are no studies which address the developmental aspect of ROS action on synaptic transmission. We have shown earlier that hydrogen peroxide (H_2O_2) caused a strong inhibition of synaptic transmission in adult mice. This effect was likely mediated by synaptosomal-associated protein 25, one of the presynaptic soluble N-ethylmaleimide-sensitive factor activating protein receptor proteins (Giniatullin et al., 2006). The neuromuscular junction is a classical model to study synaptic processes, performing reliable recordings from young, adult, and old animals. In the present study, we investigated the action of ROS on synaptic transmission at different stages of life

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http://dx.doi.org/10.1016/j.neurobiolaging.2015.10.023

(from newborns to old animals) at the neuromuscular junction. We show here a strong inhibition of transmitter release by ROS in adult and old animals with almost full resistance to ROS on spontaneous release along with the enhancement of evoked release in neonates.

2. Materials and methods

2.1. Preparation and solutions

Experiments were carried out on rat phrenic nerve—diaphragm and soleus muscle in vitro preparations from newborn (P1–P7; P0 was a day of birth), adult (P35–6 months), and old (24–30 months) rats at room temperature (~20 °C–22 °C) as these muscles show clear age-dependent changes (Brown et al., 1992; Greising et al., 2013; Imagita et al., 2009). All experiments were performed in accordance with the European Community Council Directive for the humane treatment of laboratory animals of September 22, 2010 (2010/63/EEC), and the experimental protocol was approved by the Animal Care and Use Committee of Kazan State Medical University and University "G. d'Annunzio" of Chieti-Pescara. The left diaphragm muscle was isolated together with the phrenic nerve and placed thoracic side up into the experimental chamber (2.5 mL). To prevent muscle contractions and preserve a physiologically high level of transmitter release in experiments with stimulation of the motor nerve, we used transverse cutting of muscle fibers (Glavinovic, 1979). The muscle was slightly stretched, and muscle fibers were carefully cut across their length, about 5 mm on each side of the main nerve branch. Before recording, the cut muscle was rinsed for at least 40 minutes with a basic physiological solution. The cutting procedure does not produce significant changes in cable properties and enables long-lasting stable recording of multiquantal synaptic currents (Glavinovic, 1979; Sokolova et al., 2003). Recordings of spontaneous transmitter release were performed on uncut diaphragm muscles. The basic physiological solution contained (mM): NaCl 120, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 11, $NaHPO_4$ 1, $NaHCO_3$ 24, permanently bubbled with a mixture of 95% O_2 and 5% CO_2 (starting usually 1 hour before the experiment), pH 7.3–7.4. K^+-evoked transmitter release was measured after muscles had been incubated for 10–15 minutes with solutions containing high K^+ concentrations (15 mM or 30 mM) where the increase of KCl was accompanied by the proportional reduction of NaCl.

H_2O_2 was diluted in the basic solution from 30% stock (Sigma) to obtain a concentration of 300 μM. Phorbol 12-myristate 13-acetate (PMA) and chelerythrine (both from Sigma) were prepared from 10 mM stock solutions in dimethylsulfoxide (DMSO; Tocris, Ellisville, MO, USA). The working concentration of PMA was 0.5 μM, chelerythrine—5 μM. All drugs were dissolved to the final concentrations in basic solution just before the experiments and were applied to a muscles maintained in the chamber via a superfusion system (rate ~2 mL/min) for 20–40 minutes until their full effect achievement. DMSO concentrations in applied solutions never exceeded 0.1% of the total volume. This amount of DMSO does not affect transmitter release.

2.2. Electrophysiology

Recording of evoked postsynaptic multiquantal end-plate potentials (EPPs) and spontaneous miniature end-plate potentials (MEPPs) was performed using standard glass microelectrodes (resistance 8–15 MΩ when filled with 3 M KCl). For adult and old animals, only muscle fibers with a resting membrane potential more negative than −30 mV and −60 mV were considered for evoked and spontaneous transmitter release measurement, respectively. In the cut muscles, recording started after the stabilization of membrane potential approximately 40 minutes after the cutting procedure. MEPPs at stable baseline conditions were recorded for 10–15 minutes in control and 10–15 minutes after the maximal drug effect was reached (20–40 minutes after application) in the same cells and then averaged to obtain the mean values. EPPs (elicited every 5–10 seconds by a single supramaximal phrenic nerve stimulation using a suction electrode) were collected from several individual synapses (during 2–5 minutes in every synapse) of each diaphragm before and after drug application. In newborn animals, muscle fiber membrane potentials values were lower than in adults (between −35 and −55 mV in intact muscles, and ~−22 mV in cut muscles). As the instability of membrane potential in newborns precluded long-lasting recording of synaptic events from a single fiber, we, therefore, recorded both evoked EPPs and spontaneous MEPPs from 5–12 individual synapses (for 2–5 minutes in every synapse) of each diaphragm, before and after 20–40 minutes of drug application. Only fibers in which the membrane potential dropped by less than 5 mV during the recording time were taken into consideration.

Each synaptic event was visually inspected to prevent noise disturbance of the analysis. Both EPPs and MEPPs were amplified using custom-made low-noise amplifier, digitized at 50 kHz, stored on a PC, and analyzed off-line to calculate mean amplitudes and interevent intervals (for MEPPs) using Origin 9.0 software (OriginLab Corp).

2.3. Assays for hydroperoxides

Concentrations of hydroperoxides were measured by ferrous oxidation in xylenol orange (FOX1; Deiana et al., 1999; Giniatullin et al., 2005; Jiang et al., 1992; Wolff, 1994). This method is highly sensitive and consists of peroxide-mediated oxidation of ferrous ions in an acidic medium containing the dye xylenol orange, which binds the resulting ferric ions to produce a blue-purple complex with an absorbance maximum of between 540 and 580 nm. The FOX1 reagent was prepared as described by Wolff (1994) with slight modifications: 50 mM xylenol orange, 500 μm ammonium ferrous sulphate, 200 mM D-sorbitol, and 50 mM sulphuric acid. All reagents were of at least analytic grade. The calibration curve was made using diluted 30% H_2O_2 (Ultra Pure grade). Diaphragm muscle samples were fixed in liquid nitrogen, then homogenized in cold (−20 °C) acetone and centrifuged for 10 minutes at 12,000 g. The supernatant was mixed with an equal volume of the FOX1 reagent. The reaction mixtures were incubated at room temperature for 5 minutes to enable the reaction to reach a stable end point. After the reaction was completed, the tissue extract was centrifuged for 3 minutes at 12,000 g, and the supernatant was assayed spectrophotometrically (absorbance at 560 nm). Solutions containing pure acetone mixed with an equal volume of FOX1 reagent were used as the blanks. The FOX1 reagent was made up 1 day before the analysis and kept overnight at 4 °C in the dark. All procedures were performed under dimmed light. Each measurement was made at least 3 times and then averaged. The peroxide content of samples was determined with reference to a calibration curve obtained with known concentrations of H_2O_2 and expressed as micromoles of peroxide per gram of tissue.

2.4. Antioxidant enzyme activity

The activities of various enzymes, including catalase, Se-dependent glutathione peroxidase 1 (GPX1), superoxide dismutase type 1 (SOD1), glutathione reductase (GSR), and glutathione S-transferase (μ + p) (GST μ + p), were measured in the cytosolic fractions of diaphragm muscle preparations from newborn (P7), adult (4 months), and old (24 months) rats in vitro at room