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Alessia Pascale

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Pre-exposure of Neuroblastoma Cell Line to Pulsed Electromagnetic Field Prevents H_2O_2-Induced ROS Production by Increasing MnSODActivity

Cecilia Osera,[1]* Marialaura Amadio,[1] Stefano Falone,[2] Lorenzo Fassina,[3,4] Giovanni Magenes,[3,4] Fernanda Amicarelli,[2,5] Giovanni Ricevuti,[6] Stefano Govoni,[1] and Alessia Pascale[1]

[1]*Department of Drug Sciences, Section of Pharmacology, University of Pavia, Pavia, Italy*
[2]*Department of Life, Health and Environmental Sciences, University of L'Aquila, L'Aquila, Italy*
[3]*Department of Industrial and Information Engineering, University of Pavia, Pavia, Italy*
[4]*Center for Tissue Engineering, University of Pavia, Pavia, Italy*
[5]*Institute of Translational Pharmacology, National Research Council, L'Aquila, Italy*
[6]*IDR "Santa Margherita," Department of Internal Medicine and Therapeutics, Section of Geriatrics and Gerontology, University of Pavia, Pavia, Italy*

Electromagnetic fields (EMFs) have been linked to increased risk of cancers and neurodegenerative diseases; however, EMFs can also elicit positive effects on biological systems, and redox status seems crucially involved in EMF biological effects. This study aimed to assess whether a short and repeated pulsed EMF (PEMF) could trigger adaptive responses against an oxidative insult in a neuronal cellular model. We found that a 40 min overall (four times a week, 10 min each) pre-exposure to PEMF did not affect major physiological parameters and led to a significant increase of Mn-dependent superoxide dismutase activity in the human neuroblastoma SH-SY5Y cell line. In addition, we found PEMF-pre-exposed cells exhibited decreased reactive oxygen species production following a 30 min H_2O_2 challenge, with respect to non pre-exposed cells. Our findings might provide new insights on the role played by short and repeated PEMF stimulations in the enhancement of cellular defenses against oxidative insults. Although studies in normal neuronal cells would be useful to further confirm our hypothesis, we suggest that specific PEMF treatments may have potential biological repercussions in diseases where oxidative stress is implicated. Bioelectromagnetics. 9999:1–14, 2015. © 2015 Wiley Periodicals, Inc.

Key words: electromagnetism; SH-SY5Y; oxidative stimulus; superoxide dismutase; reactive oxygen species

INTRODUCTION

Extremely low-frequency electromagnetic fields

heimer's disease [Davanipour et al., 2007; Sardi et al., 2011; Maes and Verschaeve, 2012]. On the other hand, ELF–EMF stimulation of brain nerve cells has been proposed as treatment for neurodegenerative disorders, with special attention to Alzheimer's dis-

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ease, since such stimulation improves some cognitive symptoms [Laxton et al., 2010]. In particular, ELF–EMF brain stimulation techniques, such as non-invasive transcranial magnetic stimulation, have been developed to treat cognitive dysfunctions [Cotelli et al., 2011; Di Lazzaro et al., 2013]. Pulsed electromagnetic field (PEMF) stimulation has been employed in bone tissue engineering [Fassina et al., 2006; Icaro Cornaglia et al., 2006; Fassina et al., 2009; Saino et al., 2011; Fassina et al., 2012; Ceccarelli et al., 2013], bone repair and regeneration, and in treating different types of pain, including post-surgery pain [Harden et al., 2007; Rohde et al., 2010; Hug and Rösli, 2011].

Depending on dose and timing, ELF–EMF-induced effects can be either cytotoxic or cytoprotective [Carmody et al., 2000; Gobba et al., 2009]; however, biological responses also rely on responsiveness of cell type, tissue or organism investigated [Simko, 2007; Akdag et al., 2013].

It has been suggested that ELF–EMFs affect cellular redox status, evoking a general stress response [Goodman and Blank, 2002; Simko, 2007; Falone et al., 2007] and increasing expression of stress-related proteins, such as the molecular chaperones involved in proper protein folding (heat shock proteins, e.g., HSP70) [Mannerling et al., 2010; Osera et al., 2011]. Moreover, some studies reported an increase of reactive oxygen species (ROS) production under certain ELF–EMF timing and stimulating conditions [Lupke et al., 2006; Morabito et al., 2010]. ROS overproduction may damage cellular components mainly by attacking membrane lipids and nucleic acids, or by affecting enzymatic and non-enzymatic antioxidant machinery [Simko, 2007].

On the other hand, a short ELF–EMF exposure has also been reported to prevent apoptotic death in Jurkat cells with no changes in ROS production [Palumbo et al., 2006].

Interestingly, in rat brains, an acute exposure to ELF–EMFs has been demonstrated to affect redox status mainly by increasing the antioxidant activity, thus triggering a sort of adaptive response able to preserve oxidative balance [Martinez-Samano et al., 2012]. In rats exposed to ELF–EMFs for 10 days, a significant weakening of brain cortex antioxidant defenses was shown, whereas young animals exposed to the same treatment showed positive redox enzymatic response of the antioxidative mitochondrial Mn-dependent superoxide dismutase (MnSOD), as well as an activation of neurotrophic signaling [Falone et al., 2008].

In the context of ELF–EMFs exerting a positive effect, we have previously detected in a human neuroblastoma cell line exposed to PEMF for 72 h a significant increase in chaperone HSP70 protein levels and a parallel enhanced release of a neuroprotective factor, namely the soluble amyloid precursor protein α (sAPPα) [Zhang et al., 2011; Osera et al., 2011]. Based on these findings, we hypothesized that ELF–EMFs, when properly administered in terms of dose and timing, may elicit a cytoprotective response. Cytoprotection might be the result of a sort of hormetic effect, where low doses of otherwise adverse chemical/physical stimuli improve the "functional ability" of cells and tissues to face noxae [Rattan, 2004]. This agrees with the observation that exposure to a mild stress can increase cellular defenses against harmful challenges [Calabrese et al., 2010,2011]. Here we employed SH-SY5Y human neuroblastoma cells as they exhibit many biochemical and functional properties of a neuronal cell type. Both undifferentiated and differentiated SH-SY5Y cells have been utilized for in vitro experiments requiring neuronal-like cells [Kovalevich and Langford, 2013]. Moreover, some of us previously showed that undifferentiated SH-SY5Y cells promptly respond to short-term ELF–EMF exposure by improving cellular viability and triggering significant redox–based adaptive responses [Falone et al., 2007; Sulpizio et al., 2011].

The present paper's goal was to investigate if short and repeated pre-exposure of SH-SY5Y cells to PEMF (2 mT; 75 Hz) positively affected cellular response to subsequent stress, such as hydrogen peroxide (H_2O_2). We found that pre-exposure of neuroblastoma cells to PEMF elicited a reduced ROS production associated with higher MnSOD activity following the H_2O_2 challenge.

MATERIALS AND METHODS

Electromagnetic Bioreactor

The electromagnetic bioreactor used in this study was previously described in terms of biological effects [Fassina et al., 2008; Osera et al., 2011; Fassina et al., 2012] and in terms of dosimetry and induced electric field [Mognaschi et al., 2014]. In order to rule out the possibility of any thermal effect beyond the in silico study [Mognaschi et al., 2014], temperature of the culture medium in PEMF- and sham-exposed samples was checked and no PEMF-induced heating was detected (data not shown). Briefly, the apparatus was assembled in a custom-machined carrying structure within a tube of polymethylmethacrylate. The windowed tube carried two solenoids with parallel planes. The cells were placed 5 cm away from each solenoid plane and the

solenoids were powered by a pulse generator (BIOSTIM SPT, Igea, Carpi, Italy). In this experimental setup, magnetic field and induced electric field were perpendicular and parallel to the seeded cells, respectively. The PEMF had the following parameters: intensity, 2 ± 0.2 mT; frequency, 75 ± 2 Hz; pulse duration, 1.3 ms. The electromagnetic bioreactor was placed into the cell culture incubator and cells were exposed for different times. Non-PEMF pre-exposed cultures were placed into a different incubator in the absence of PEMF stimulation.

Cell Cultures and Treatments

were fixed with 4% paraformaldehyde (Sigma-Aldrich, Milan, Italy), stained with 1 mg/ml of Brilliant Blue (Sigma-Aldrich, Milan, Italy), and dissolved in methanol 50% and acetic acid 7.5%, as previously described [Marchesi et al., 2014]. Colonies with more than 50 cells were counted by using the Clono-counter software developed by Niyazi et al. [2007] (software was downloaded from the paper's supplementary material).

Caspase 3/7 Enzymatic Activity

Apoptosis was monitored with the Caspase-Glo 3/7 Assay kit, following manufacturer instructions

5% CO_2 and 95% humidity. Cells were pre-exposed to PEMF for 30 to 15 or 10 min, for a total of 4 treatments over 7 days, or continuously for 72h. After 24h of PEMF stimulation, PEMF-exposed and non-PEMF pre-exposed cells (hereafter called pre-exposed and non pre-exposed, respectively) were treated with 1 mM H_2O_2 (Sigma-Aldrich, Milan, Italy) for 10 or 30 min.

MTT Assay

Mitochondrial function was estimated using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma-Aldrich, Milan, Italy). Briefly, 1.25×10^3 cells/well were seeded into 96-well plates. After treatments, 10 µl of MTT (final concentration of 1 mg/ml) was added to each well. After 4 h at 37 °C, formazan crystals were solubilized in 100 µl of lysis buffer (20% sodium dodecyl sulfate in 50% dimethylformamide) overnight at 37 °C. Absorbance values were measured at 595 nm in a microplate reader (model 550, Bio-Rad Laboratories, Milan, Italy) and results expressed as arbitrary units (AU).

Cell Counting

Cells (5×10^4/well) were seeded into 6-well plates and pre-exposed to PEMF or not, as described above, and then trypsinized and counted in a Bürker hemocytometer chamber, using a 1:2 dilution in 0.04% Trypan Blue (Sigma-Aldrich, Milan, Italy). Results were expressed as viable cell number/ml.

Clonogenic Survival Assay

Cells (1.5×10^3/well) were plated into 6-well plates. Then, cells were either treated or not with PEMF. After 2 weeks from PEMF exposure, cells ized to the cell number.

Western Blotting Analysis

Cells were homogenized as previously described [Amadio et al., 2012], and total protein content was measured via Bradford's method [Bradford, 1976]. Denatured proteins (10 µg) were separated by 12% SDS–polyacrylamide gel electrophoresis and processed as previously described [Solerte et al., 1998]. Anti-HSP70 mouse monoclonal antibody (dil. 1:1,000), anti-PKCα mouse monoclonal antibody (dil. 1:750), and anti-HuR mouse monoclonal antibody (dil. 1:1,000) were purchased from Santa Cruz Biotechnology (Dallas, TX); whereas rabbit polyclonal anti-MnSOD (dil. 1:2,000) was supplied by Enzo Lifescience (Vinci-Biochem, Florence, Italy). Rabbit polyclonal anti-BCL2 (dil. 1:1,000) was from Cell Signaling (Danvers, MA), and mouse monoclonal anti-α-tubulin (dil. 1:1,000) was purchased from Sigma-Aldrich (Milan, Italy). All antibodies were diluted in T–TBS buffer [10 mM Tris–HCl, 100 mM NaCl, 0.1% (v/v) Tween 20, pH 7.5], containing 6% (v/v) skimmed milk. Signals on nitrocellulose membranes were detected by chemiluminescence (Thermo Fisher Scientific, Waltham, MA). Experiments were performed in duplicate for each different cell preparation, using α-tubulin as the loading control and for data normalization. Images were digitally acquired and processed using NIH Image software (http://rsb.info.nih.gov/nih-image).

Immunoprecipitation Assay

Cells were washed twice with ice cold phosphate buffered saline without calcium and magnesium

(PBS), homogenized and processed according to previously published protocol [Amadio et al., 2008]. Briefly, immunoprecipitation was performed overnight at 4 °C using 1 µg of anti-HuR IgG (Santa Cruz Biotechnology, Dallas, TX) for 50 µg of protein sample diluted in 1 ml volume of immunoprecipitation buffer [150 mM NaCl, 50 mM Tris–HCl (pH 7.4), 1 mM $MgCl_2$, 0.05% Nonidet P-40, and a protease inhibitor cocktail], in the presence of 50 µl of protein A/G plus agarose (Santa Cruz Biotechnology, Dallas, TX) previously blocked with 5% bovine serum albumin in the same buffer. The obtained pellets underwent Western blotting using mouse antibody anti-phosphothreonine (dil. 1:400; Santa Cruz Biotechnology, Dallas, TX). The negative control was obtained under identical conditions, but in the presence of an irrelevant (IRR) antibody of the same isotype of the specific immunoprecipitating antibody. Small aliquots of immunoprecipitation mixes were collected from each sample and used as "input signals" to normalize Western blotting data.

Detection of Reactive Oxygen Species

ROS formation was measured by using the fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH–DA; VWR International, Milan, Italy) which forms 2′,7′-dichlorofluorescein when oxidized by ROS. Briefly, cells were seeded into a 96-well plate (1.25×10^3 cells/well) and kept upon PEMF or not. After H_2O_2 treatment, cells were washed with PBS, loaded with DMSO-resuspended 10 µM DCFH–DA, and placed in the incubator for 30 min. Cells were washed with PBS and the fluorescent intensity was measured after 30 min with SpectraMax Gemini microplate spectrofluorometer (λ_{exc} 485 nm; λ_{emi} 530 nm) (model XS; Molecular Devices, Wokingham, United Kingdom). Results were expressed as percentages of controls. ROS production was normalized on the cell viability parameter.

Tissue Homogenate Preparation for Enzymatic and Protein Assays

Sub-confluent control and treated cells (4×10^7 cells/ml) were harvested and resuspended in 0.1 M phosphate buffer, pH 7, containing 0.1% (v/v) Triton X-100. Cell suspensions were homogenized and centrifuged at 13,000 × g for 30 min at 4 °C. The resulting supernatant was used for spectrophotometric measurement of both enzymatic activity and protein content [Bradford, 1976].

Superoxide Dismutase Activity

its ability to inhibit auto-oxidation of epinephrine, which was determined by monitoring the increase in absorbance at 480 nm at 30 °C [Sun and Zigman, 1978]. An appropriate amount of supernatant was used to obtain about 50% inhibition of the epinephrine auto-oxidation in a 50 mM $NaHCO_3$ (pH 10.2) buffer, in the presence of 0.1 mM EDTA (all from Sigma-Aldrich, Milan, Italy). In order to discriminate between copper–zinc (Cu–ZnSOD) and manganese-containing (MnSOD) superoxide dismutase enzyme activities, the assay was repeated after incubation with 0.4 volumes of chloroform: ethanol (15:25) to remove the MnSOD-related contribution. MnSOD specific activity was derived by monitoring and determining spectrophotometrically the enzymatic activities of total SOD (tSOD) and Cu–ZnSOD [Paynter, 1980; Falone et al., 2008]. All biochemical assays were performed as blind experiments, and results given as enzymatic specific activities.

Data Analysis

All statistical analyses were performed using GraphPad InStat application (GraphPad software, Prism 6, La Jolla, CA). All data were analyzed using one-way or two-way analysis of variance (ANOVA), and, when appropriate, a specific post-hoc test, as indicated in figure legends. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Repeated 10 min PEMF Stimulation Did Not Alter Basic Cellular Functions

We compared effects of continuous, or short and repeated PEMF pre-exposure on mitochondrial activity in SH-SY5Y cells. As already shown in our previous study [Osera et al., 2011], we confirmed that 72 h PEMF pre-exposure (Fig. 1a) dramatically decreased mitochondrial function in human neuroblastoma cells (- 34%; Fig. 1b). Then, we focused on short PEMF stimuli of 30, 15 or 10 min, repeated 4 times over a period of 7 days (PEMF overall stimulations: 2 h, 1 h or 40 min, respectively; Fig. 1c). Both 30 and 15 min PEMF exposure significantly reduced mitochondrial activity with respect to non-exposed cells, whereas the MTT assay did not detect any effect induced by 10 min PEMF exposure (Fig. 1d). Cell proliferation, clonogenic survival and the key components of the apoptotic pathway were not significantly altered by