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Power frequency magnetic fields induced reactive oxygen species-related autophagy in mouse embryonic fibroblasts

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ABSTRACT

Power frequency magnetic fields (PFMF) have been reported to affect several cellular functions, such as cell proliferation and apoptosis. In this study, we investigated the effects of PFMF on mouse embryonic fibroblasts (MEF) autophagy. After cells were exposed to 50 Hz PFMF at 2 mT for 0.5 h, 2 h, 6 h, 12 h, and 24 h, we observed a significant increase in autophagic markers at 6 h, including (i) higher microtubule-associated protein 1 light chain 3-II (LC3-II), (ii) the increased formation of GFP-LC3 puncta, and (iii) increased numbers of autophagic vacuoles under transmission electron microscope. Moreover, we provide convincing evidence using chloroquine (CQ) that the increase of autophagic markers was the result of enhanced autophagic flux and not the suppression of lysosomal function. In a search for molecular mechanisms underlying PFMF-mediated autophagy, we observe that the autophagic process involved reactive oxygen species (ROS) and was independent of the mammalian target of rapamycin (mTOR) signaling pathway.

1. Introduction

With the development of the electric industry and the application of electric technology, human beings are inevitably exposed to 50 or 60 Hz PFMF, which are generated by alternating current supplied by, for instance, power lines and domestic appliances. PFMF have been classified as possibly carcinogenic to humans (Group 2B) by the International Agency for Research on Cancer (IARC, 2002). The potential effects of PFMF on human health are a matter of public concern and a significant amount of research has been conducted. However, the World Health Organization (WHO) has claimed that the scientific evidence supporting a linkage between PFMF and diseases is weak (2010), and the biological effects induced by PFMF is somewhat contradictory, whereas the mechanism remains unclear (Santini et al., 2009). Further studies are therefore urgently needed. The limit values for public and occupational exposure to PFMF are 0.2 mT and 1 mT, respectively (2010). Therefore, we choose an intensity, which is beyond these limit values to study the biological effects of PFMF.

As an environmental factor, PFMF have been reported to affect various cellular functions. PFMF can induce DNA double-strand breaks in human lung fibroblast cells, apoptosis, and cell cycle arrest in prostate cancer cells (Kim et al., 2010a; Koh et al., 2008). PFMF induced the activation of EGFR-sensitive actin cytoskeleton motility in FL cells (Wu et al., 2014), and resulted in a transient increase of myoblast migration (Iorio et al., 2013). In contrast, PFMF have been reported to alter the homeostasis of Ca^{2+} in neural tissues (Manikonda et al., 2007; Sert et al., 2011), increase ROS in different cell lines (Koh et al., 2008; Park et al., 2013), enhance antioxidant defenses and induce a shift in redox homeostasis in neuroblastoma cells (Falone et al., 2007). In summary, PFMF may induce alterations of various cellular functions by disturbing homeostasis.

Autophagy is now recognized as indispensable for the homeostasis of cells, tissues and organisms (Hubbard et al., 2012; Marino et al., 2011; Meijer and Codogno, 2004). Macroautophagy (hereafter referred to autophagy) is a dynamic biological process in which cytosol and organelles are sequestered within double-membrane vesicles known as autophagosomes that deliver the contents to the lysosome/vacuole for degradation and recycling of the resulting macromolecules to optimize the usage of limited energy (Klionsky, 2005). Autophagy serves to reduce oxidative damage (Scherz-Shouval and Elazar, 2011), which is vital in physiological and

Abbreviations: PFMF, power frequency magnetic fields; MEF, mouse embryonic fibroblasts; LC3-II, microtubule-associated protein 1 light chain 3-II; RAPA, rapamycin; CQ, chloroquine; ROS, reactive oxygen species; mTOR, mammalian target of rapamycin; IARC, International Agency for Research on Cancer; WHO, World Health Organization; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate buffered saline; BSA, bovine serum albumin; RT, room temperature; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DCFH-DA, 2′-7′-dichlorodihydrofluorescein diacetate; 7AAD, 7-amino-actinomycin D; DAPI, 4′, 6-diamidino-2-phenylindole; p70S6K, p70S6 kinase.

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Table 1
Percentage of apoptotic cells in each group.

Group	Viable	Apoptotic
Sham	94.78 ± 2.32	3.55 ± 2.57
Exposure	94.20 ± 3.14	4.39 ± 3.08
Positive solution	3.18 ± 2.41	74.97 ± 16.19*

pathological situations including neonatal starvation, the degradation of disease-causing aggregate-prone proteins and the clearance of pathogenic bacteria (Ravikumar et al., 2010). In this study, we investigated the effects of 2 mT PFMF on autophagy in MEF.

2. Materials and methods

2.1. Exposure system

An extremely low frequency magnetic field exposure system, which has been described in detail by other groups(Schuderer et al., 2004), was designed by the Foundation for Information Technologies in Society (sXc-ELF, IT'IS Corporation). The complete system included the coil chambers, a signal tower, a PC with controlling software, and a suitable CO_2 incubator. Two chambers in one cell culture incubator (Heraeus) can ensure constant environmental conditions (37 °C, 5% CO_2). Each chamber is composed of a series Helmholtz coil providing 50 Hz power-frequency current by a controlled current. The coil in one chamber is in-phase connected to generate an enhanced magnetic field for a vertical electromagnetic field-exposed group, whereas an opposite phase connection in another chamber generates offset magnetic field for sham-exposed group. The flux densities for powerline mode range from 0.02 to 2.3 mT and the intensity described in this paper was 2 mT. More detailed information may be found on the website of IT'IS Foundation. (http://www.itis.ethz.ch)

2.2. Cell culture

MEF were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Gibco) at 37 °C in a humanized atmosphere of 5% CO_2 incubator (Thermo Scientific). The MEF stably express GFP-LC3 after the addition of 400 ng/ml G418 (Gibco) into the culture medium (Hosokawa et al., 2007). One day prior to exposure to PFMF, cells were seeded onto tissue culture dishes (Corning) at a final density of 8×10^4 cells/ml or into 96-well tissue culture plates (Thermo Scientific) at a final density of 5×10^4 cells/ml.

2.3. Antibodies and reagents

Rabbit anti-LC3 antibody and mouse anti-β-actin antibody were purchased from Sigma-Aldrich. Rabbit anti-p70s6k antibody and mouse anti-pospho-p70s6k antibody were from Cell Signaling Technology. Goat-anti-rabbit IRDye 800cw and goat-anti-mouse IRDye 680cw were form LI-COR Biosciences. Rapamycin (RAPA) and chloroquine were purchased from Sigma-Aldrich.

2.4. Western blotting

Western blotting analysis was performed following standard procedures (Aldridge et al., 2008). The cells were lysed in RIPA lysis buffer (Beyotime) that combined with phosphatase inhibitor cocktail (Sigma) and protease inhibitor mixture (Roche Applied Science). The protein concentration was determined by the BCA Protein Assay Kit (Beyotime). An equal amount of protein for each sample was separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred onto PVDF membrane (Bio-Rad) at 200 mA for 2 h in transfer buffer. After blocking with 5% BSA, the sample was incubated with a primary antibody at room temperature (RT) for 2 h or at 4 °C overnight and then incubated with a secondary antibody at RT for 1 h. Finally, the PVDF membrane was detected using the Odyssey Infrared Imaging System (LI-COR).

2.5. Transmission electron microscopy

After treatments, autophagosomes were observed using TEM according to standard procedures (Eskelinen, 2008). Cells were trypsinized and centrifuged at 200*g* for 3 min. After being resuspended in 10% bovine serum albumin (BSA), cells were pelleted at 200*g* for 3 min. The BSA was removed and cells were fixed with 2.5% glutaraldehyde at 4°C overnight. The pellets were washed in PBS (phosphate buffered saline) three times followed by fixing in 1% osmium tetroxide at RT for 1 h. Next, pellets were washed in water twice and stained in 2% uranyl acetate at RT for 1 h. Pellets were dehydrated by 70% ethanol for 15 min, 90% ethanol for 15 min, 100% ethanol twice for 15 min and finally 100% acetone once for 20 min. Samples were infiltrated into a mixture of resin and propylene oxide (1:1) at RT for 2 h and then pure resin overnight. The pellets were embedded into an embedding mixture (DDSA, MNA, DMP-30 and EPON812) and incubated at RT for 4–6 h. The samples were sectioned and dyed with uranyl acetate and lead citrate. Finally, the samples were observed by TECNAI G20 transmission electron microscopy (Field Emission Inc.).

2.6. Laser confocal scanning microscopy

Cells were seeded onto a glass coverslip in 35 mm dish (Thermo Scientific) for 24 h. After exposure, cells were immediately fixed in 4% paraformaldehyde at 4 °C for 15 min. The coverslip was washed in PBS three times and mounted onto a glass slide. The samples were examined with an Olympus IX81-FV1000 confocal microscope, equipped with a 60× oil immersion objective. GFP-LC3 puncta in the cells were quantified. Over 50 cells were randomly selected from each treatment to calculate the average number of GFP-LC3 puncta per cell.

2.7. Fluorometric assay

Intracellular ROS levels were measured using a Reactive Oxygen Species Assay Kit (Beyotime). Cells were seeded in 96-well culture plates (Corning), which are specifically designed for fluorescence assays and incubated at 37°C for 24 h. After treatments, the cells were washed three times by FBS-free DMEM. 2′-7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) at a final concentration of 10 μM was added and mixed thoroughly. The samples were then incubated at 37 °C for 30 min in the dark. After being washed three times with DMEM, the relative levels of fluorescence were quantified by a Varioskan Flash spectral scan multimode plate reader (Thermo Scientific, excitation: 488 nm, emission: 525 nm).

2.8. Flow cytometry

Apoptosis analysis was performed following standard procedures (eBioscience). After treatments, cells were washed twice in cold PBS. Next, cells were trypsinized and centrifuged at 200 g for 3 min, and then resuspended in cold binding buffer to a concentration of 10^6–10^7 cells/ml. One hundred microliters of cells was added to each tube, followed by 5 μl of Annexin V-PE and 10 μl of 7-amino-actinomycin D (7AAD). Samples were mixed gently and incubated on ice for 15 min in the dark. Without washing, 380 μl