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Infrasonic noise induces axonal degeneration of cultured neurons via a Ca^{2+} influx pathway

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HIGHLIGHTS

- Infrasonic noise induces axonal degeneration.
- Infrasound-induced axonal degeneration precedes neuronal cell death.
- Ca^{2+} influx involves in the process of infrasound-induced axonal degeneration.

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ABSTRACT

Infrasound is a kind of environmental noise. It can evoke biological resonance in organismic tissues including the central nervous system (CNS), causing displacement and distortion of cellular architectures. Several studies have revealed that certain intensity infrasound can impair normal functions of the brain, but the underlying mechanisms still remain largely unknown. Growing evidence has demonstrated that axonal degeneration is responsible for a variety of CNS dysfunctions. To explore whether neuronal axons are affected under infrasonic insults, we exposed cultured hippocampal neurons to infrasound with a frequency of 16 Hz and a pressure level of 130 dB for 1 h, and examined the morphological and molecular changes of neuronal axons by immunocytochemistry and Western blotting, respectively. Our results showed that infrasound exposure significantly resulted in axonal degeneration of cultured hippocampal neurons, which was relatively independent of neuronal cell death. This infrasound-induced axonal degeneration can be significantly blocked by Ca^{2+} chelator EGTA and Rho kinase inhibitor Fasudil, but not by proteasome inhibitor MG132. Moreover, calcium imaging and RhoA activation assays revealed a great enhancement of Ca^{2+} influx within axons and RhoA activation after infrasound exposure, respectively. Depletion of Ca^{2+} by EGTA markedly inhibited this Ca^{2+} influx and attenuated RhoA activation as well. Thus, our findings revealed that axonal degeneration may be one of the important mechanisms underlying infrasound-induced CNS impairment, and Ca^{2+} influx pathway is likely implicated in the process.

1. Introduction

Infrasound is a kind of public low frequency noise with oscillation frequency below 20 Hz (Alves-Pereira and Castelo Branco, 2007). As an acoustic stimulation, undue exposure to infrasound can induce extra-aural and even whole-body pathology known as vibroacoustic disease, which compromises multiple organs of the body. For example, it causes thickening of cardiovascular structures, depression, aggressiveness, increased irritability and decreased cognitive skills. (Castelo-Branco and Alves-Pereira, 2004). Although the underlying mechanisms of infrasound-induced dysfunction remain largely unknown, a body of evidence suggests that infrasonic wave can trigger biological resonance (Dommes et al., 2009; Pei et al., 2007; Shi et al., 2003). All the human organs have their own inherent vibration frequencies (e.g., the head, 8–12 Hz; the thoracic cavity, 4–6 Hz; the heart, 5 Hz; the abdominal cavity, 6–9 Hz), which are just within the range of infrasound frequency (0–20 Hz). Therefore, infrasound with certain frequency

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and sound pressure will induce intensive organ resonance, which can cause mechanical displacement and distortion of tissues or even individual cells (Backteman et al., 1984).

The central nervous system (CNS) is vulnerable to infrasonic insults. It is reported that some aircraft technicians who work daily in infrasonic environment show an abnormal brain magnetic resonance imaging pattern or a fairly high incidence of late-onset epilepsy (Castelo Branco and Rodriguez, 1999). Moreover, other CNS symptoms such as dizziness, severe vertigo, unique and sudden episodes of non-convulsive neurological deficit and delay in multimodal evoked potentials, are also frequently observed in these people (Castelo-Branco and Alves-Pereira, 2004; Castelo Branco and Rodriguez, 1999). Our group has been exploring the mechanisms underlying infrasound-induced impairments. We previously found that long-term infrasound exposure could induce neuronal apoptosis of hippocampal cells in rat brain (Liu et al., 2004a), which may be resulted from an increase in Ca^{2+} influx (Liu et al., 2004b). However, our results also revealed that this apoptosis was not evident after short-term infrasound exposure (unpublished data). These findings lend support to our hypothesis that in addition to neuronal cell death, other mechanism(s) may be involved in infrasound-induced CNS disorders.

Recently, growing evidence suggests that active axonal degeneration is responsible for a variety of CNS dysfunctions (Coleman and Perry, 2002; Finn et al., 2000; Song et al., 2006). In contrast to passive axonal degeneration resulting from cell death, active axonal degeneration is independent of neuronal cell death, and is often finely regulated by a group of molecules. Axons growing from cell bodies often navigate over long distances and cover broad field before reaching their targets. Therefore, it is expected that compared with the cell bodies, the axons may be more susceptible to various insults, such as injury, toxins, inflammation and ischemia. Mechanisms underlying axonal degeneration are not fully understood. Recent observations show that Ca^{2+} overload is one of the key points responsible for axonal degeneration (George et al., 1995; Glass et al., 1994). Axotomy, axon dynamic stretch, vincristine or beta-amyloid induction and NGF deprivation (Buki and Povlishock, 2006; Kilinc et al., 2009; Song et al., 2006; Zhai et al., 2003), can result in Ca^{2+} accumulation within axons and subsequently activate Ca^{2+}-dependent cysteine protease calpain, which finally hydrolyzes microtubule-based cytoskeleton (Billger et al., 1988; Johnson et al., 1991; Kilinc et al., 2009). Microtubule, a major constituent of the cytoskeleton, is of great importance in maintaining axon integrity. Its destabilization and disassembly is not only one of the earliest detectable axonal events but also sufficient to cause axonal degeneration (Luduena et al., 1986; Wang et al., 2001; Zhai et al., 2003). Loss of microtubules can impair axonal transport and result in an axonal beading morphology (Maxwell and Graham, 1997). In addition to Ca^{2+} influx, the RhoA/Rho kinase (ROCK) signaling pathway is reported to be involved in axonal degeneration due to its essential role in microtubule destabilization (Luo and O'Leary, 2005). Inhibition of the RhoA/ROCK pathway prevented axonal degeneration following methylmercury exposure or axotomy (Fujimura et al., 2011; Yamagishi et al., 2005). Finally, a previous study showed that the ubiquitin–proteasome system (UPS) is also involved in active axonal degeneration. After axotomy in the optic nerve and the axons of cultured superior cervical ganglion neurons, UPS inhibitor MG132 was found to delay the process of microtubule fragmentation (Zhai et al., 2003).

To explore whether axonal degeneration occurs under infrasonic insults, in the present study, we exposed cultured hippocampal neurons to infrasound with a frequency of 16 Hz and a pressure level of 130 dB and examined possible changes of neuronal axons. Our data showed that infrasound exposure caused significant axonal degeneration, which seemed to be independent of cell death. Moreover, we revealed that Ca^{2+} signaling pathway may be involved in this process.

2. Materials and methods

2.1. Infrasound device and parameters

The infrasound device used in the present study was described in detail in our previous study (Du et al., 2010). Infrasound of a frequency of 16 Hz and sound pressure level of 130 dB was adopted on the basis of our previous studies (Du et al., 2010; Liu et al., 2010; Shi et al., 2008). One hour of infrasound exposure was used in the study, based on our preliminary experiments, in which different exposure times (0.5, 1, and 2 h) were examined and 1-h exposure was found to be optimal for the observation of gradual change of axons (data not shown). Hence, dishes containing cultured cells were placed in the infrasonic chamber and subjected to 16 Hz, 130 dB infrasound for 1 h. During exposure, the frequency and pressure level of infrasound were monitored by a data collection system. The control cells were maintained in the same chamber for 1 h without infrasound exposure.

2.2. Cell culture

Primary culture of hippocampal neurons was performed as described previously (Kaech and Banker, 2006). Briefly, the hippocampi of E18–E19 Sprague Dawley rat embryos were minced with forceps, digested with 0.25% trypsin and homogenized in DMEM containing 10% FBS (Gibco/BRL, Gaithersburg, MD, USA). After centrifugation at $200 \times g$ for 5 min, the pellets were resuspended in Neurobasal (Gibco/BRL) containing 2% B27 (Gibco/BRL), 1% glutamine (Sigma–Aldrich Co., St. Louis, MO, USA), 100 μg/ml streptomycin, and 100 U/ml penicillin. The dissociated cells were plated at a density of 5×10^3 cells/cm^2 onto glass coverslips (for immunocytochemical assays) or 1.5×10^5 cells/cm^2 in 60 mm dishes (for Western blotting assays) coated with poly-D-lysine (Sigma–Aldrich). The medium was changed every 3 days. All experiments were performed ten days after cell plating.

2.3. Drug application

ROCK inhibitor Fasudil (10 μM, Tocris Cookson Ltd., Bristol, UK) and UPS inhibitor MG132 (20 μM, Calbiochem, Darmstadt, Germany) were added to the medium 12 h and 3 h prior to infrasound exposure, respectively (Lingor et al., 2007; Zhai et al., 2003). Calcium chelator EGTA (2 mM, Sigma) and calpain antagonist ALLN (50 μM, Calbiochem) were added 1 h before infrasound exposure (Zhai et al., 2003). These drugs were also administered to the cultured neurons without infrasound exposure, serving as the controls.

2.4. Immunocytochemistry

The cultured cells at 0, 4, 8, 12 or 24 h post exposure to infrasound were fixed with 4% paraformaldehyde for 30 min at room temperature. After washing with PBS, the cells were incubated with the following primary antibodies at 4 °C overnight: mouse anti-β-III-tubulin (Chemicon, Temecula, CA, USA), rabbit anti-cleaved caspase-3 (Cell Signaling Technology, Beverly, MA, USA), rabbit anti-β-amyloid precursor protein (anti-APP, Zymed-Invitrogen, Carlsbad, CA, USA) and rabbit anti-Glu-tubulin (Chemicon). For Glu-tubulin immunostaining, soluble cytoplasmic proteins within neurons were extracted in 0.1 M phosphate buffer, containing 10 mM EGTA, 2 mM $MgCl_2$ (pH 6.9), 4% paraformaldehyde, and 0.2% Triton X-100 for 30 min before staining (Mimura et al., 2006). After incubation with species-specific secondary antibodies conjugated to Cy2 or Cy3 (Jackson ImmunoResearch, West Grove, PA, USA) for 2 h at room temperature, the fluorescent signals were visualized under a fluorescence microscope (Leica GmbH, Wetzlar, Germany) or a confocal laser microscope (Olympus, Center Valley, PA, USA). The nuclei were counterstained with 1 μg/ml Hoechst 33342 (Sigma-Aldrich).

2.5. Cell death analysis

For evaluation of apoptosis after infrasound exposure, TUNEL staining was performed as described by the manufacturer (Roche, Indianapolis, IN, USA) and nuclei were stained with Hoechst 33342. For necrosis detection, propidium iodide (PI) staining was performed (Unal Cevik and Dalkara, 2003). In brief, cultured neurons were washed three times with PBS and then incubated with 1 μM PI (Sigma-Aldrich) for 20 min at 37 °C. TUNEL- or PI-stained cells were observed and counted under a fluorescence microscope.

2.6. Western blot analysis

Total cellular proteins were extracted from cultured neurons as previously described (Mimura et al., 2006). Denatured proteins were loaded in equal amounts to each lane, subjected to SDS–PAGE and transferred onto polyvinylidene fluioride membrane (Millipore, Bilerica, MA, USA). After incubation in blocking buffer (Tris-buffered saline containing 5% defatted milk powder) at room temperature for 1 h, the membranes were blotted with mouse anti-β-III-tubulin, rabbit anti-Glu-tubulin and rabbit cleaved caspase-3 antibodies at 4 °C overnight. Rabbit anti-β-actin (Santa