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● *Original Contribution*

APOPTOSIS INDUCED BY MICROBUBBLE-ASSISTED ACOUSTIC CAVITATION IN K562 CELLS: THE PREDOMINANT ROLE OF THE CYCLOSPORIN A-DEPENDENT MITOCHONDRIAL PERMEABILITY TRANSITION PORE

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Abstract—Acoustic cavitation of microbubbles has been described as inducing tumor cell apoptosis that is partly associated with mitochondrial dysfunction; however, the exact mechanisms have not been fully characterized. Here, low-intensity pulsed ultrasound (1 MHz, 0.3-MPa peak negative pressure, 10% duty cycle and 1-kHz pulse repetition frequency) was applied to K562 chronic myelogenous leukemia cells for 1 min with 10% (v/v) SonoVue microbubbles. After ultrasound exposure, the apoptotic index was determined by flow cytometry with annexin V–fluorescein isothiocyanate/propidium iodide. In addition, mitochondrial membrane potential ($\Delta\Psi_m$) was determined with the JC-1 assay. Translocation of apoptosis-associated protein cytochrome c was evaluated by Western blotting. We found that microbubble-assisted acoustic cavitation can increase the cellular apoptotic index, mitochondrial depolarization and cytochrome c release in K562 cells, compared with ultrasound treatment alone. Furthermore, mitochondrial dysfunction and apoptosis were significantly inhibited by cyclosporin A, a classic inhibitor of the mitochondrial permeability transition pore; however, the inhibitor of Bax protein, Bax-inhibiting peptide, could not suppress these effects. Our results suggest that mitochondrial permeability transition pore opening is involved in mitochondrial dysfunction after exposure to microbubble-assisted acoustic cavitation. Moreover, the release of cytochrome c from the mitochondria is dependent on cyclosporin A–sensitive mitochondrial permeability transition pore opening, but not formation of the Bax-voltage dependent anion channel complex or Bax oligomeric pores. These data provide more insight into the mechanisms underlying mitochondrial dysfunction induced by acoustic cavitation and can be used as a basis for therapy. (E-mail: fengyi@mail.xjtu.edu.cn or mxwan@mail.xjtu.edu.cn) © **2015 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Microbubble, Acoustic cavitation, Mitochondrial permeability transition pore, Cytochrome c, Cyclosporin A.**

INTRODUCTION

Acoustic cavitation is the rapid expansion, contraction and/or collapse of bubbles driven by cyclic change of the pressure of an ultrasound field and can be used to concentrate energy and forces onto a small volume in a variety of biomedical applications (Brujan 2004; Nomikou and McHale 2010; Paliwal and Mitragotri 2006; ter Haar 2007). Microbubbles have been reported to enhance biological and chemical effects by acting as cavitation nuclei (Miller and Thomas 1995). When acoustic cavitation takes place in the vicinity of living cells, microbubbles in cell suspensions can oscillate vigorously or collapse, which may lead to cell injury (Guzman et al. 2001; Lai et al. 2006; Schlicher et al. 2010). A series of physical and chemical mechanisms are associated with this phenomenon. Microstreaming and concurrently generated local shear stress are potential mechanisms underlying the creation of pores on the cell membrane (Collis et al. 2010; Park et al. 2011; Tran et al. 2007; Wu 2002). Moreover, reactive oxygen species (e.g., ·OH radicals and ·H atoms from sonochemical decomposition of water) may participate in a variety of cellular events, such as DNA degradation, inactivation of enzymes, lipid peroxidation, membrane damage and cell apoptosis (Hassan et al. 2009; Juffermans et al. 2006; Riesz and Kondo 1992).

Microbubble-assisted acoustic cavitation has been found to cause cell cycle arrest, morphologic repression, development delays and cellular apoptosis (Feril et al.

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2003; Firestein et al. 2003; Miller et al. 2003; Zhong et al. 2011). Several types of microbubbles (e.g., SonoVue, Levovist, Albunex and Optison) have been reported to enhance the apoptotic effects induced by acoustic cavitation (Ando et al. 2006; Juffermans et al. 2009; Ward et al. 1999, 2000). Moreover, the activation of the intrinsic mitochondrial apoptosis pathway caused by mitochondrial dysfunction is considered a major contributory process in cellular apoptosis underlying microbubble-assisted acoustic cavitation (Feril et al. 2005; Honda et al. 2002, 2004). Mitochondrial dysfunction can induce opening of the mitochondrial permeability transition pore (mPTP) and cause mitochondrial swelling through the release of apoptosis-inducing factors into the cytoplasm such as cytochrome c (Cyt c), which facilitates formation of the apoptosome and initiates the caspase cascade, ultimately leading to programmed cell death *via* apoptosis (Jiang and Wang 2000; Lemasters et al. 2009); however, whether mPTP opening is involved in acoustic cavitation-induced apoptosis remains uncertain.

The mPTP is a proteinaceous megapore putatively consisting of cyclophilin D (Cy D) in the matrix, adenine nucleotide translocator (ANT) in the mitochondrial inner membrane and voltage-dependent anion channel (VDAC) in the mitochondrial outer membrane (Shimizu et al. 1999; Yang and Cortopassi 1998). Cyclosporin A (CsA) is regarded as a potent inhibitor of permeability transition because it prevents the binding of Cy D to ANT, thereby preventing ANT from changing into the pore-forming c-conformation (Hausenloy et al. 2002; Tsujimoto and Shimizu 2007). Moreover, the pro-apoptotic protein Bax can increase the opening of VDAC by forming the Bax–VDAC complex or form a pore in the mitochondrial outer membrane on oligomerization (Narita et al. 1998). In fact, there are two main pathways closely involved in the induction of Cyt c release from the mitochondria, a CsA-sensitive progress and a CsA-insensitive Bax-associated process (Naranmandura et al. 2012; Weiss et al. 2003); however, there is little information regarding which potential mPTP pathway is the main mediator of the release of Cyt c from the mitochondria during microbubble-assisted acoustic cavitation.

Here, we studied the effects of acoustic cavitation with microbubbles on mitochondria to determine the signaling pathway underlying opening of the mPTP and release of Cyt c in human leukemia K562 cells. Microbubbles significantly increased acoustic cavitation-induced apoptotic effects, mitochondrial depolarization and Cyt c release from mitochondria compared with the ultrasound exposure alone group. These mitochondrial dysfunctions were strongly suppressed by CsA, but not significantly by a Bax-inhibiting peptide. Furthermore, CsA inhibited the apoptotic effects induced by acoustic cavitation. These findings provide the first evidence that microbubble-assisted acoustic cavitation could open the mPTP mainly through a CsA-sensitive pore to induce Cyt c release from mitochondria and promote cellular apoptosis, while the Bax-associated pathway may not participate. The above investigations could clarify additional molecular mechanisms of mitochondrial dysfunction induced by microbubble-assisted acoustic cavitation.

METHODS

K562 cell line culture

Human leukemia K562 cells were obtained from the Center of Laboratory Animals of the Fourth Military Medical University. The cells were grown in suspension cultures in RPMI-1640 medium supplemented with 10% fetal calf serum and 100 units/mL penicillin–streptomycin. K562 cells were incubated at 37°C in a humidified CO_2 (5%) incubator.

Pre-exposure preparations

In each experimental trial, a K562 cell suspension was first transferred to the wells of a six-well plate (Corning-Costar, Corning, NY, USA). Just before ultrasound exposure, the cell suspension was pretreated with 1 μM CsA (Wako Pure, Osaka, Japan) or 2 μM Bax-inhibiting peptide (Calbiochem, Merk, Darmstadt, Germany) for 20 min, and then the microbubbles were added. SonoVue (Bracco, Milan, Italy), used as artificial gas nuclei, is a suspension of stabilized sulfur hexafluoride (SF_6) microbubbles encased in a phospholipid monolayer shell with a diameter of approximately 2.5 μm. The original concentration, approximately 10^8 microbubbles/mL, was diluted 100-fold with physiologic saline to a final density of 10^6 microbubbles/mL before ultrasound exposure. A microbubble solution (10% v/v) was added to the cell suspension for each exposure trial. In our experiments, the cell-to-microbubble ratio was approximately 10:1, which was previously found, in a microbubble dosage study, to efficiently cause cell injury without inducing large numbers of cell lysis (Guzmán et al. 2003). For the well comparison, experiments were carried out using sham-treated cells that were subjected to the same conditions as the ultrasound-treated cells, except that neither ultrasound nor microbubbles were applied during the exposure time. Our preliminary experiments indicated that there are no significant differences in cellular viability and survival between the presence and absence of microbubbles under the culture conditions of the sham-treated cells.