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Cardiolipin plays a role in KCN-induced necrosis

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ABSTRACT

Cardiolipin (CL) is a unique anionic, dimeric phospholipid found almost exclusively in the inner mitochondrial membrane and is essential for the function of numerous enzymes that are involved in mitochondrial energy metabolism. While the role of cardiolipin in apoptosis is well established, its involvement in necrosis is enigmatic. In the present study, KCN-induced necrosis in U937 cells was used as an experimental model to assess the role of CL in necrosis. KCN addition to U937 cells induced reactive oxygen species (ROS) formation, while the antioxidants inhibited necrosis, indicating that ROS play a role in KCN-induced cell death. Further, CL oxidation was confirmed by the monomer green fluorescence of 10-*N*-nonyl acridine orange (NAO) and by TLC. Utilizing the red fluorescence of the dimeric NAO, redistribution of CL in mitochondrial membrane during necrosis was revealed. We also showed that the catalytic activity of purified adenosine triphosphate (ATP) synthase complex, known to be modulated by cardiolipin, decreased following KCN treatment. All these events occurred at an early phase of the necrotic process prior to rupture of the cell membrane. Furthermore, CL-deficient HeLa cells were found to be resistant to KCN-induced necrosis as compared with the wild type cells. We suggest that KCN, an effective reversible inhibitor of cytochrome oxidase and thereby of the respiratory chain leads to ROS increase, which in turn oxidizes CL (amongst other membrane phospholipids) and leads to mitochondrial membrane lipid reorganization and loss of CL symmetry. Finally, the resistance of CL-deficient cells to necrosis further supports the notion that CL, which undergoes oxidation during necrotic cell death, is an integral part of the milieu of events taking place in mitochondria leading to membrane disorganization and mitochondrial dysfunction.

1. Introduction

Cardiolipin (CL) is a unique anionic phospholipid with a dimeric structure. It contains two phosphates (two negative charges), three glycerols and four unsaturated fatty acid acyl chains. The fatty acyl chain composition of CL is highly specific, being predominantly comprised of 18-carbon unsaturated acyl chains, the vast majority of which in most mammalian tissues is linoleic acid (18:2) (Hoch, 1992; Schlame et al., 2005). However, the degree of unsaturation can vary among the different tissues. In the mammalian heart 18:2 constitutes 80–90% of CL acyl chains, and tetralinoleoyl CL (L_4CL) is the most abundant species (Chicco and Sparagna, 2007). Other studies showed about 74–80% of symmetric tetralinoleoyl CL in heart and ~30% in neutrophils. It was shown that in lymphocytes the most abundant CL species are 18:2 (Valianpour et al., 2005).

In mammalian cells, cardiolipin is found almost exclusively in the inner mitochondrial membrane (Fernandez et al., 2002). The role of mitochondria (and particularly of cardiolipin) in regulation of apoptosis has been discovered in many cell types (Gonzalvez et al., 2008). HeLa cells, where CL levels were manipulated by knocking down CL synthase showed increased resistance to apoptosis induced by actinomycin D along with decreased CL peroxidation and cytochrome c release (Huang et al., 2008a). It was shown that the preceding event that leads to CL peroxidation is the increase in intracellular reactive oxygen species (ROS) which is one of the early events of apoptosis induced by a variety of agents (Orrenius and Zhivotovsky, 2005). Huang et al. (Huang et al., 2006) showed that the amount of cardiolipin was inversely correlated with reactive oxygen species generation and correlated with mitochondrial membrane potential. Later it was shown that

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hyperoxia-induced increase in ROS generation was correlated with the amount of lipid oxidation products (Huang et al., 2008b).

CL is also essential for the optimal function of numerous enzymes that are involved in mitochondrial energy metabolism, such as NADH:ubiquinone, oxidoreductase, cytochrome c oxidase, ADT/ATP carrier protein, adenosine triphosphate (ATP) synthase and the cytosolic proteins i.e., tBid (Lutter et al., 2000; Gohil et al., 2004; Mileykovskaya et al., 2005; Claypool et al., 2008; Wenz et al., 2009; Epand et al., 2002). Barth syndrome is a mitochondrial disorder caused by an inborn error of phospholipid metabolism. Tafazzin deficiency inhibits specifically the acyl remodeling of cardiolipin. As a result, mitochondria contain reduced levels of cardiolipin and the remaining cardiolipin lacks its characteristic acyl pattern (Schlame and Ren, 2006; Gebert et al., 2009). The hydrophobic double-unsaturated linoleic diacylglycerol species seem to be required for the high affinity binding of cardiolipin to proteins (Cao et al., 2004). Several observations showed the link between oxidation of CL and cytochrome c release. Cytochrome c binds preferentially to CL, a mitochondrial lipid, but not to cardiolipin hydroperoxide (CL-OOH) (Nomura et al., 2000; Kriska et al., 2005a). In addition, the GSH/selenoperoxidase-over-expressing clone of COH-BR1 breast cancer cells was strongly resistant to apoptosis compared to the parent cells (Kriska et al., 2005a). These facts indicate that lipid peroxidation in mitochondrial membranes can induce cytochrome c release. This statement gains support from the fact that CL peroxidation, catalyzed by cytochrome c, is required for cytochrome c release from mitochondria (Kagan et al., 2005).

For a long time necrosis, a type of mammalian cell death was considered, in contrast to apoptosis, a passive, uncontrolled and unorganized way of cell death. Recent studies suggest that necrosis is tightly controlled and includes programmed processes such as mitochondrial dysfunction, enhanced generation of reactive oxygen species, ATP depletion and early plasma membrane rupture (Prabhakaran et al., 2002; Moquin and Chan, 2010; Cho et al., 2010). It was shown in different cell culture systems that common components of both apoptotic and necrotic programs are activated when various inducers (e.g., cytokines, heat, irradiation and pathogens) stimulate signaling pathways that participate in both processes in the same cell population (Proskuryakov et al., 2003). ROS increase in both apoptosis and necrosis. In apoptosis, the increase in intracellular ROS, which is one of the early events of apoptosis, leads to CL peroxidation (Orrenius and Zhivotovsky, 2005). However, the role of CL in necrosis is hitherto unknown. CL was found in the ATP synthase complex at stoichiometric ratio and links proteins within the complex (Eble et al., 1990) and its peroxidation could affect ATP synthase activity in necrosis. Furthermore, it was suggested that the regulation of cardiolipin distribution in the mitochondrial membrane involves the existence of an active cardiolipin transport process in the inner membrane, which is mediated by mitochondrial ATP synthesis and could be affected by decrease in ATP levels (Petit et al., 1994).

All these led us to investigate whether CL could be involved in necrosis. Our study shows that in addition to KCN inhibition of the mitochondrial respiratory chain, elevated levels of ROS could induce CL peroxidation and its redistribution. Thus treating cells with a necrotic stimulus such as KCN leads to mitochondrial membrane lipids reorganization that probably results from partial redistribution of CL molecules into the outer leaflet. The changes in CL distribution may indicate the biological role of lipid asymmetry and lipid exchange between monolayers in normal condition and during cell death. This is further supported by the resistance of CL-deficient cells to necrosis.

2. Material and methods

2.1. Cell culture

U937 cells were cultured in RPMI, supplemented with 10% heat-inactivated fetal bovine serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin, at 37 °C in a 5% CO_2 humidified atmosphere. HeLa cells (wild type or TAZ-1 mutant HeLa cells where CL levels were manipulated by knocking down CL synthase and obtaining ~45% of normal CL content) were plated at a density of 1×10^5/ml and cultured at 37 °C in a 5% CO_2 humidified atmosphere in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (FBS), 2 mM L-glutamine, 2 mM sodium pyruvate, 0.4 IU/ml penicillin, 0.4 mg/ml streptomycin, 0.02 mg/ml neomycin, 0.5 mg/ml G418. Cells were passaged regularly and subcultured to ~80% confluence before experimental procedures.

2.2. Necrosis/apoptosis-treatment and analysis assays

Necrosis in U937 cells (4×10^5 cells/ml) was induced by 5 and 10 mM KCN (1 M stock solution in water) following 1 h preincubation in glucose-free RPMI fortified with pyruvate. This experimental system was previously described by us (Zelig et al., 2009). When required, pre incubation with *N*-acetyl-cysteine (NAC) or glutathione for 45 min before KCN addition was done. Apoptosis was induced by 7 h treatment of U937 cells with 1.25 μM staurosporine (STS), in complete RPMI. STS is a broad-range kinase inhibitor that is known to be a potent apoptosis inducer. The biological activity of staurosporine is the inhibition of protein kinases through the prevention of ATP binding to the kinase. This is achieved through the stronger affinity of staurosporine to the ATP-binding site on the kinase. Staurosporine is a prototypical ATP-competitive kinase inhibitor in that it binds to many kinases with high affinity, though with little selectivity. Necrosis in HeLa cells was induced following 1 h preincubation in glucose-free DMEM, and thereafter treating the cells with up to 30 mM KCN for 24 h. To differentiate between apoptotic and necrotic cell death, cells were stained with acridine orange (AO) and ethidium bromide (EB). Cultured cells were centrifuged at 650 g for 10 min and stained with AO and EB at a final concentration of 0.05 mg/ml each. One hundred cells were counted from each sample to evaluate the death mode using a fluorescence microscope as previously described by us (Hallak et al., 2008, 2009).

Cell viability was assessed by diluting an aliquot of cell suspension with an equal volume of 0.4% trypan blue solution. After staining with trypan blue solution, viable (unstained) cells were counted in a hemocytometer chamber. CytoTox 96®, the Non-radioactive Cytotoxicity Assay kit (Promega, Madison, WI) was used to determine lactate dehydrogenase (LDH) release.

Apoptotic cell death was determined by propidium iodide (PI) labeling followed by cell cycle analysis. The cells were harvested by centrifugation, washed once with ice-cold PBS and fixed in 70% ethanol at 4 °C overnight. The cells were then washed once with ice-cold PBS and re-suspended in PBS (pH 7.4) containing 0.1% Triton X-100 and 10 μg/ml RNase at room temperature for 40 min. Finally 15 μg/ml PI was added to the cells and they were analyzed by flow cytometry on a Beckman Coulter Cytomics FC 500 5-color flow cytometer equipped with an argon ion laser (488 nm), using CXP software to determine the percentage of the apoptotic cells (Hallak et al., 2008, 2009).