Phospholipid signaling in apoptosis: peroxidation and externalization of phosphatidylserine

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Abstract

The role of phospholipids in apoptosis signaling and the relationship between oxidation of phosphatidylserine and its redistribution in the plasma membrane were studied. A novel method for detection of site-specific phospholipid peroxidation based on the use of cis-parinaric acid as a reporter molecule metabolically integrated into membrane phospholipids in living cells was employed. When several tissue culture cell lines and different exogenous oxidants were used, the relationship between the oxidation of phosphatidylserine and apoptosis has been revealed. The plasma membrane was the preferred site of phosphatidylserine oxidation in cells. It was shown that selective oxidation of phosphatidylserine precedes its translocation from the inside to the outside surface of the plasma membrane during apoptosis. A model is proposed in which cytochrome c released from mitochondria by oxidative stress binds to phosphatidylserine located at the cytoplasmic surface of the plasma membrane and induces its oxidation. Interaction of peroxidized phosphatidylserine with aminophospholipid translocase causes inhibition of the enzyme relevant to phosphatidylserine externalization. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Phospholipids are the dominant lipid constituents of the membranes of animal cells where they are arranged in a bilayer configuration and act as the matrix for the support and organization of the different membrane proteins. In addition to
this structural role many individual phospholipid constituents are known to be involved in specific signaling functions necessary for cells to respond to external stimuli. Such signals invariably involve redistribution and/or metabolism (hydrolysis, oxidation) of the target phospholipid, the products of which either modulate protein functions directly or are further converted to products that serve this function (Ohanian et al., 1998; Wilton, 1998; Malisan et al., 1999). Phosphoinositol-dependent pathways and eicosanoid cascades are two prominent examples of the important role(s) that phospholipids play in cell physiology (De Jonge et al., 1996; Martin, 1998; Versteeg et al., 1999).

More recently, lipid metabolism and turnover have been shown to be associated with initiation and/or progress of cell death. Metabolism of ceramides, for example, is known to be a factor culminating in apoptosis (Mathias et al., 1998; Perry and Hannun, 1998). Oxidation of the unsaturated fatty acyl residues of phospholipids, such as formation of lipid peroxides, can lead either to apoptosis or necrotic cell death depending on the particular stimulus and the extent of phospholipid involvement (Smith 1987; Pacifici et al., 1994a; Glaesser et al., 1996; Kaneko and Baba, 1999). While there is strong evidence that oxidative stress, in general, and lipid peroxidation, in particular, are involved in both initiation and mediation of apoptosis, the specific mechanism(s) that translate peroxidation of one or more classes of phospholipids into the recognizable biochemical and morphological stages of apoptosis remains unclear.

Recent studies have provided evidence that membrane phospholipid asymmetry is disturbed in one of the early stages of apoptosis (Reno et al., 1998). Specifically, a translocation of phosphatidylserine (PS) from the internal surface to external surface of the plasma membrane appears to be a fundamental mechanism by which apoptotic cells are recognized and eliminated by phagocytic macrophages (Fadok et al., 1992; Hampton et al., 1996; Fadok et al., 1998). PS externalization could arise either through inactivation of aminophospholipid translocase, whose normal function maintains the asymmetric distribution of PS in cells (Verhoven et al., 1995; Bruckheimer and Schroit, 1996) or by accelerated reversal of movement of the phospholipid. Aminophospholipid translocase is a membrane-bound ATP-dependent enzyme whose normal role is to transport aminophospholipids, phosphatidylethanolamine (PE) and PS, from the external to the internal leaflet of the plasma membrane. Inhibition of this enzyme results in disruption of membrane phospholipid asymmetry and exposure of PS, to the outside of the cell (Martin and Pagano, 1987). Another enzyme, phospholipid scramblase, transports multiple phospholipids, including PS bidirectionally. Activation of this enzyme is required to initiate the loss of membrane asymmetry in apoptosis (Bratton et al., 1997).

Another promising line of investigation appears to be to link a disturbance of mitochondrial electron transport in the early stages of apoptosis with the generation of free radicals the action of which results in peroxidation of membrane phospholipids, increased mitochondrial permeability and release of cytochrome c (Mignotte and Vayssiere, 1998). Apoptotic events are known to include cytochrome c release from mitochondria and its accumulation in cytosol. This is associated with activation of caspase-9, which then leads to processing and activation of other capases (Green and Reed, 1998).

Over the past few years our research group has been examining the hypothesis that susceptibility of PS to oxidation is augmented by binding of basic proteins, in particular cytochrome c, which are released from mitochondria and bind to the acidic phospholipid. Oxidation of PS, in turn, is an early step in the process of apoptosis.

2. Measurements of phospholipid peroxidation in live cells

Experimental approaches to demonstrate involvement of phospholipid oxidation in cell homeostasis and signaling processes have proved to be relatively difficult because of inherent problems of quantitating phospholipid peroxidation in living cells. This is largely due to a remarkably efficient remodeling/repair of oxidized phospholipids. For example, phospholipid hydroperoxides have long been known to be efficient substrates
Fig. 1. Protective effect of bcl-2 transfection on oxidation of parinaroyl-phosphatidyserine and apoptosis induced by AMVN in PC12 pheochromocytoma cells. (A) AMVN induced oxidation of parinaroyl-phosphatidyserine in mock- and bcl-2-transfected PC12 pheochromocytoma cells. Cells pre-labeled with PnA were incubated with different concentrations of AMVN for 2 h at 37°C then lipids were extracted and resolved by HPLC. (B) AMVN induced apoptosis in mock- and bcl2-transfected PC12 pheochromocytoma cells. Cells were incubated for up to 100 min in the presence of 0.75 mM AMVN. Percentage of cells demonstrating apoptosis was manually determined for each of three high-power fields using fluorescence microscopy of acridine orange–ethidium bromide stained cells. All data are means ± S.E.M. *P < 0.05.

Fig. 2. Effect of phenol and AMVN on oxidation of phospholipids and induction of apoptosis in normal human epidermal keratinocytes. (A) Oxidation of phospholipids in normal human epidermal keratinocytes induced by phenol. PnA pre-labeled keratinocytes were incubated in the presence of phenol (50 μM) for 2 h at 37°C, then lipids were extracted and resolved by HPLC. (B) Oxidation of phospholipids in normal human epidermal keratinocytes induced by AMVN. PnA pre-labeled keratinocytes were incubated in the presence of AMVN (500 μM) for 2 h at 37°C, then lipids were extracted and resolved by HPLC. (C) Effect of phenol and AMVN on the appearance of hypodiploid keratinocytes. Keratinocytes were incubated with phenol (50 μM) or AMVN (500 μM) for 2 h at 37°C then apoptosis (number of hypodiploid cells) was determined by flow cytometry. All data are means ± S.E.M.
Fig. 3. Normal-phase HPLC chromatograms of total lipids extracted from plasma membrane of (A) control HL60 cells and (B) HL60 cells treated with tert-BuOOH. Fluorescence emission intensity, (excitation at 324 nm, emission at 420 nm) was measured in the column effluent. Cells were incubated in the presence and absence of tert-BuOOH (150 μM) for 20 min and then BHT (10 μM) was added and the subcellular organelles were isolated. Lipids from the plasma membrane were extracted and resolved by HPLC. PnA, cis-parinaric acid; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; SPH, sphingomyelin.

for endogenous phospholipases and particularly phospholipase A₂ (Kagan, 1988). More recent studies have shown that accumulation of phospholipid hydroperoxides in membranes not only stimulates phospholipase A₂-catalyzed hydrolysis of hydroperoxy-containing molecular species but accelerates hydrolysis of non-oxidized phospholipids in membranes (Salgo et al., 1992). Subsequent reacylation of monoacylglycerophospholipids is also stimulated by phospholipid peroxidation thereby augmenting phospholipid turnover. Thus, acylcarnitine-dependent reacylation of lysophospholipids is elevated many-fold as a consequence of oxidative stress (Pacifici et al., 1994b). Such phospholipid remodeling masks potential role(s) that oxidatively modified phospholipids may play in apoptotic pathways.

To address these problems we have developed a novel method of detecting phospholipid oxidation based on the use of cis-parinaric acid (PnA) as a reporter molecule for lipid peroxidation in living mammalian cells (Ritov et al., 1996). PnA is a naturally-occurring, 18-carbon fatty acid containing four conjugated double bonds; it has a conformation similar to that of other polyunsaturated fatty acid residues normally associated with membrane phospholipids (Welti, 1982). Oxidative destruction of any part of the conjugated double bond system of PnA results in the disappearance of its characteristic fluorescence at 420 nm (Kuypers et al., 1987) so that measurements of fluorescence emission intensity can be used to monitor lipid oxidation. PnA can be metabolically incorporated into different classes of membrane phospholipids of cells cultured under physiological conditions (Rintoul and Simoni, 1977) and used as a sensitive and specific reporter system to measure oxidative stress in membranes in living cells. This method has proved to be successful in demonstrating the involvement of oxidation of different phospholipid classes in living cells subjected to oxidative stress using a variety of oxidants (Ritov et al., 1996; Kagan et al., 1998). It is, therefore, possible to detect oxidation of specific
phospholipids with a level of precision sufficient to identify potential involvement of phospholipid metabolism in cell physiological events.

3. Relationship between phospholipid peroxidation and apoptosis

The response of cells in tissue culture to oxidative stress has been assessed by exposing cells containing metabolically labeled parinaroyl-phospholipids to exogenous oxidants and measuring the susceptibility of different phospholipids to oxidation. A lipid-soluble azo-initiator, 2,2'-azo-bis(2,4-dimethylvaleronitrile) (AMVN), is a preferred source of free radicals for such experiments because of its unique ability to generate peroxyl radicals exclusively within the hydrophobic environment of membranes (Niki, 1990; Krainev and Bigelow, 1996). When HL60 cells pre-labeled with PnA were exposed to AMVN, lipid peroxidation could be easily detected from the fluorescence of individual phospholipid classes resolved by HPLC. It was found that exposure to AMVN resulted in oxidation of all PnA-labeled phospholipids in HL60 cells, but in an amount that was beyond the level that could be detected as a change in phospholipid composition of the cells. Exposure to AMVN also induces apoptosis in these cells as evidenced by (i) DNA fragmentation, (ii) appearance of apoptotic nuclei, and (iii) externalization of PS (Fabisiak et al., 1998a). Thus, the nuclear phenotype in cells exposed to AMVN is characterized by chromatin condensation, fragmentation and internucleosomal DNA cleavage. This is associated with significant changes in the distribution of PS in the plasma membrane such that asymmetry tends to be lost and the phospholipid appears on the surface of the cell. There is also a strong correlation between the appearance of annexin V/propidium iodide positive HL60 cells and the oxidative stress induced by exposure to AMVN (Fabisiak et al., 1998a).

It follows that if oxidative stress is related to initiation or mediation of apoptosis then protection against such stress should arrest the process. The major lipid-soluble antioxidant within cell

![Fig. 4. A model scheme of the role of early cytochrome c release from mitochondria in oxidation and externalization of phosphatidylserine in the plasma membrane.](image-url)
membranes is vitamin E (Burton and Ingold, 1989). The ability of antioxidants and antioxidant enzymes to protect cells from apoptosis has been suggested previously (Hockenbery et al., 1993; Kane et al., 1993). To investigate the role of antioxidants as possible protectors against oxidative stress, a homologue of vitamin E, such as 2, 2,5,7,8-pentamethyl-6-hydroxy chromane (PMC), was used to determine the extent of protection provided against AMVN-induced peroxidative modification of specific classes of phospholipids in the membranes of HL60 cells. It is well known that PMC is an efficient lipid antioxidant that randomly protects lipids from oxidation (Kagan et al., 1990). In HL60 cells exposed to AMVN, PMC greatly attenuated the loss of PnA fluorescence in phosphatidylcholine (PC), PE, and phosphatidylinositol (PI), but oxidation of PS was not prevented by the antioxidant. In model experiments in which liposomes prepared from PnA-labeled phospholipids of HL60 cells were exposed to AMVN, substantial oxidation of all phospholipids, similar to that seen in intact cells, was demonstrated. However, in this cell-free system PMC was fully able to protect all phospholipids, including PS.

The significance of this failure of PMC to protect PS from oxidation in HL60 cells oxidatively stressed by exposure to AMVN was apparent from the progress of apoptosis in cells treated with PMC. The presence of PMC during exposure of the HL60 cells to AMVN failed to block the formation of DNA ladders and other AMVN-induced apoptotic changes in nuclear morphology. Moreover, externalization of PS following treatment with the azo initiator of peroxyl radicals was also unaffected by the presence of PMC consistent with flow cytometry measurements of annexin V binding to externalized PS (Fabisiak et al., 1998a).

4. Control of phospholipid peroxidation at the level of gene expression

Bcl-2 is one of a family of proteins that can inhibit apoptosis. These proteins have also been shown to protect membrane lipids from peroxidation during exposure to oxidative stress (Hockenbery et al., 1993; Kane et al., 1993). In PC12 pheochromocytoma cells exposed to AMVN a uniform pattern of peroxidation of PnA-labeled membrane phospholipids is observed and this is associated with a concentration dependent induction of apoptosis (Tyurina et al., 1997). The specific rate of oxidation was found to be greater for PS than for any of the other phospholipid classes (Fig. 1). Products of the bcl-2 gene protect PC12 cells against apoptosis induced by oxidative stress and prevent oxidation of all the phospholipids, including PS, during exposure of cells to relatively low concentrations of AMVN. It is noteworthy that protection of PS oxidation by bcl-2 gene products was significantly less than that of the other phospholipid classes and also that full protection against apoptosis was not observed (Tyurina et al., 1997). Thus Bcl-2 only partly protects PS against oxidation and does not completely prevent apoptosis induced by AMVN in PC12 cells.

5. Responses to different oxidative stresses

The relationship between oxidation of PS and redistribution of the phospholipid in the plasma membrane is central to our understanding of the role of the phospholipid in apoptosis. The use of a variety of different exogenous oxidants to explore the relationship between oxidative stress, PS and apoptosis has been particularly revealing. The use of different oxidants has suggested that the form and type of radicals involved may influence the type of response observed in a particular cell line.

Experiments using paraquat as the source of oxidative stress in 32D cells have, for example, clearly shown that selective oxidation of PS precedes its translocation from the inside to the outside surface of the plasma membrane and apoptosis (Fabisiak et al., 1997). Apoptosis in response to paraquat is characterized by chromatin condensation and fragmentation, internucleosomal DNA cleavage, and loss of cell viability within 24 h of toxin exposure. Paraquat induced early and selective oxidation of PnA, primarily in PS, which was subsequently followed by apopto-
sis. PS peroxidation preceded its appearance on the cell surface by several hours. Overexpression of Bcl-2 afforded significant protection against paraquat-induced apoptosis as well as peroxidation of PnA metabolically integrated into PS. Quinacrine, similarly to Bcl-2, attenuated the selective oxidation of PS and blocked paraquat-induced apoptosis in 32D cells (Fabisiak et al., 1998b).

The protection afforded by Bcl-2 against apoptosis also differs depending on the type of oxidative stress. As with the effect of oxidative stress induced by AMVN treatment, exposure of mock-transfected PC12 cells to the antitumor drug, neocarzinostatin, results in peroxidation of all membrane phospholipid classes. The proportion of apoptotic nuclei in cells following exposure to neocarzinostatin was, however, greater in bcl-2-transfected compared with mock-transfected cells (Cortazzo and Schor, 1996). It is evident that in these cells Bcl-2 does not provide protection of cells against apoptosis induced by this antitumor drug and, in contrast to AMVN, overexpression of Bcl-2 does not protect PS from oxidation or the appearance of PS on the cell surface induced by neocarzinostatin (Schor et al., 1999).

Glutamate-induced cytotoxicity is mediated primarily through necrosis. Neither PC12 nor PC12/bcl-2 cells underwent apoptosis in response to cytotoxic doses of glutamate. Significant oxidation of PnA-labeled PE and PC and their protection by products of the bcl-2 gene were observed in PC12 cells treated with glutamate (15 mM), but even with such high concentrations of glutamate there was no significant oxidation of parinaroyl-PS in these cells (Tyurin et al., 1998).

Phenol alone caused substantial oxidation of PnA-labeled phospholipids in normal human epidermal keratinocytes. Two major phospholipids — PE and PC were primary targets for peroxidation in keratinocytes (Fig. 2). PS was found to be resistant to phenol-induced oxidation and the morphology of treated keratinocytes does not show differences from that of control cells (Shvedova et al., 2000). In contrast, a significantly increased number of apoptotic cells and oxidation of all phospholipids, including PS, were detected after treatment of normal human epidermal keratinocytes with AMVN (Shvedova et al., 2000). Moreover, exposure of human keratinocytes to cumene hydroperoxide caused both enhanced peroxidation of PS and its externalization documenting execution of the apoptotic program in the cells (Shvedova et al., in preparation).

Another organic hydroperoxide, tert-BuOOH, produced site-specific oxidative stress in cardiomyocyte membrane phospholipids. The oxidant caused loss of parinaroyl-PS while other phospholipids remained unchanged (Gorbunov et al., 1998). Interestingly, tert-BuOOH selectively oxidized PS the oxidation and externalization on the cell surface of which may be associated with apoptosis in cardiomyocytes (Maulik et al., 1998). It is noteworthy that in the presence of the NO-donor, PAPANONOate, the oxidative effect of tert-BuOOH was no longer observed. Hence, the protective effect of NO may be interpreted as the ability of NO to prevent tert-BuOOH-induced apoptosis in cardiomyocytes (Gorbunov et al., 1998). This, however, is not always the case. In HL60 cells treatment with the NO donor, PAPANONOate, completely protected all phospholipids, including PS, from oxidation induced by AMVN, but it did not inhibit externalization of PS and had no effect on other markers of apoptosis following AMVN (Fabisiak et al., 2000).

In summary, our results clearly demonstrate that oxidative modification of PS may be a common pathway in apoptotic signaling in cells challenged with oxidants. Two important questions are: (i) does peroxidation of PS occur specifically in plasma membranes, and if so (ii) what are the mechanism(s) through which oxidants induce site-specific oxidation of PS? In our preliminary experiments we isolated different subcellular organelles from PnA-labeled HL60 cells to determine whether PS is indeed the preferred peroxidation substrate in plasma membranes. We found that in HL60 cells challenged with tert-BuOOH oxidation of PS was maximal in plasma membranes (Fig. 3) and endoplasmic reticulum membranes. In these organelles PS peroxidation exceeded the oxidation of other classes of phospholipids by more than 2-fold. Importantly, tert-BuOOH caused apoptosis in HL60 cells as evidenced by PS externalization and the appearance of condensed
apoptotic nuclear morphology. If one assumes that cytochrome \( c \) released from mitochondria into cytosol is involved in the catalysis of PS oxidation then it seems likely that enhanced PS oxidation would be confined to the cytotoxic surfaces of the plasma membrane and the endoplasmic reticulum membranes. This model emphasizes the central role of the early release of cytochrome \( c \) from mitochondria not only as one of the apoptosis-initiating factors but also as a catalyst of PS peroxidation related to inhibition of aminophospholipid translocase and subsequent PS externalization. An overall scheme linking release of cytochrome \( c \) from mitochondria with PS transformation in the plasma membrane is presented in Fig. 4. Future studies are in progress to examine this model.

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