Enzymes that Regulate Lipid Metabolism in Cell Signalling

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Sphingolipids related to apoptosis from the point of view of membrane structure and topology

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Abstract

Sphingolipids and their metabolites are implicated in signal transduction, but the mechanisms are still poorly understood. In particular, the presumed function of ceramide as a second messenger remains controversial. Here, we emphasize the importance of both ceramide and sphingomyelin for membrane structure. The effects of sphingolipid turnover in the induction and effector phases of apoptosis are explained by their impact on membrane microdomains that are relevant for cell signalling or changes in morphology. The topology of sphingolipid metabolism is important because of their limited transbilayer and intermembrane movement. For instance, glycosyl ceramide synthase converts de novo synthesized ceramide to glycosyl ceramide, but it is neither a general attenuator of ceramide accumulation at the plasma membrane, nor of the apoptotic process. Synthetic alkyl-lysosphospholipids modulate membrane-lipid composition and, therefore, apoptosis sensitivity.

Ceramide: second messenger in apoptosis?

Of all the sphingolipids, ceramide (Cer) has received the most attention because it is almost universally generated during cellular stress and apoptosis, either by an acid or a neutral sphingomyelinase (a/nSMase) or by de novo synthesis [1,2]. The knowledge that synthetic, short-chain Cer can induce apoptosis has led to the oversimplified extrapolation that Cer formation is an obligatory step in the apoptotic process. The main reason why this issue is still controversial [3–6] is because the molecular basis of Cer production is in many cases unresolved. It is, therefore, often not possible to abrogate Cer formation by genetic means and to examine the consequences for apoptosis induction. Several possible intracellular target molecules for Cer have been proposed, such as the protein phosphatases, PPI and PP2A, and the protein kinases, protein kinase Cζ, kinase suppressor of Ras (KSR) and Raf-1 [1,7], but these have not been verified by follow-up studies. Thus, unlike diacylglycerol and some of the phosphoinositides, Cer has not been convincingly validated as lipid second messenger. Although Cer and diacylglycerol have a similar structure, which has been suggestive for a similar binding to cysteine-rich C1 domains of protein kinases [7], their occurrence, location and structural behaviour in membranes is quite different [5] (see below), which could well explain some of the controversies in the field.

Key words: ceramide, raft, sphingomyelin, glucosylceramide synthase.
Abbreviations used: ALP, alkyl-lysosphospholipid; a/nSMase, acid/neutral sphingomyelinase; Cer, ceramide; GCS, glucosylceramide synthase; PC, phosphatidyl choline; SM, sphingomyelin; TNFα, tumour necrosis factor α.

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Part of the confusion in the literature on the significance of Cer formation also stems from the fact that there are at least two phases of Cer formation. Some investigators are able to detect rapid, transient elevation of Cer levels, minutes after the delivery of an apoptotic stimulus, while, in addition, slow and sustained Cer formation is generally observed in the effector phase of apoptosis, i.e. secondary to caspase activation [4,8–10]. As we will discuss, the cause and consequences of these two responses are completely distinct.

Ceramide as determinant of membrane structure: the induction phase

Through intermolecular hydrogen bonding and its two long, saturated hydrophobic chains, Cer fits better than diacylglycerol in a lipid bilayer structure, and thereby segregates preferentially into lateral microdomains (rafts/caveolae) where it is packed tightly in homodimers/multimers, as well as with cholesterol and the other sphingolipids (see [5,11–14] and references therein). In fact, substantial amounts of Cer can be measured in isolated rafts/caveolae, even from unstimulated cells [13,14]. For the same physico-chemical reason, natural Cer (not short-chain Cer) does not transfer spontaneously between lipid bilayers, and it is unlikely to ‘flip-flop’ as readily as diacylglycerol. It might, therefore, be restricted to the side of the bilayer on which it is generated [5,15]. Thus, if it is generated from the major pool of sphingomyelin (SM) in the exoplasmic side of membrane rafts (or rather at the luminal side of recycling endosomes; see below), Cer is unlikely to act as a rapidly generated second messenger for a cytoplasmic target protein at the opposite side of the membrane [15]. However, it could modify subtle intermolecular interactions within and among lipid rafts with consequences for raft clustering and (apoptotic) signalling.

Indeed, Gulbins and co-workers [16–18] recently provided evidence for this view. They showed that Cer that has been generated in or exogenously inserted into the outer leaflet of the raft bilayer can facilitate clustering of the CD95/Fas death-receptor and the subsequent induction of apoptosis under suboptimal concentrations of cross-linking ligand or antibodies. Their data suggest that this Cer is generated by a CD95-activated aSMase that translocates to the plasma membrane where it becomes exposed at the outer leaflet of the raft bilayer (at the cell surface) [17]. Since aSMase is a glycoprotein [6], this makes sense, but with its low pH optimum, the enzyme is unlikely to be activated there at neutral pH. An attractive idea, therefore, is that this aSMase is located in recycling endosomes, where it becomes transiently activated at low pH, generating Cer from SM at the luminal side, and upon fusion with the plasma membrane, Cer will then appear in the outer leaflet of the plasma membrane [17]. While there, it facilitates raft clustering and the patching and capping of CD95 receptors [16–18]. The involvement of an aSMase in this process was demonstrated by using aSMase-deficient cells, in which addition of natural Cer could overcome the decreased sensitivity to CD95 ligation. This new model features Cer as a membrane structural component rather than a second messenger.

It should be noted that the altered sensitivity to CD95 stimulation in the absence of aSMase only holds for certain cell types, e.g. hepatocytes, but not for others, such as primary T-lymphocytes or B-lymphoblasts [19–22]. It is a subtle phenomenon that is only observed at suboptimal concentrations of agonistic anti-CD95 antibody [16,22]. The CD95-induced, aSMase-mediated Cer production is a rapid event (occurring within seconds) and occurs locally (in rafts). If basal Cer levels in these rafts are too high (which may easily be the case [13,14]), stimulated rapid Cer production may escape detection and may also no longer be effective in facilitating CD95 clustering and apoptosis induction. As a cautionary note, aSMase−/− mice develop a Niemann–Pick-type disease and their cells have many abnormalities [6,21]. These include altered T-cell receptor responses, a severely altered composition of sphingolipids and cholesterol in their membranes, and a defect in the formation of functional lipid rafts, which can no longer be isolated by the standard procedures based on detergent insolubility [21]. One can imagine that general alterations in lipid composition rather than defective Cer formation may determine apoptosis sensitivity in such cells. We and others [20] have found that aSMase deficiency or overexpression did not affect CD95-, radiation- or cancer drug-induced apoptosis in B-lymphoblastoid cells. Clearly, aSMase activity is, in general, not essential for the induction of apoptosis, but it seems to facilitate this process in some cases.

Sphingomyelin as determinant of membrane structure: the effector phase

The deficiency in aSMase did not affect the slow and sustained Cer formation that is generally
observed in cells undergoing apoptosis [19]. We concluded that this Cer, produced downstream from inducer caspase activation in Jurkat T cells stimulated via CD95 or by γ-radiation or etoposide, results from SM hydrolysis by an undefined nSMase activity [23,24]. The function of nSMase(s) in apoptosis is difficult to assess, mainly because the enzyme, which acts in the plasma membrane and which probably also localizes in rafts/caveolae [14], has not been cloned. The function of the isozyme that has been cloned, nSMase1 [25], is unknown [6]. It localizes in the endoplasmic reticulum and/or nucleus, and we and others found that its expression/overexpression in cells does not affect Cer formation during apoptosis or apoptosis itself (see [6,26], and references therein).

We addressed the mechanism of the slow Cer formation further and found that, during apoptosis, scrambling of membrane phospholipids (loss of bilayer asymmetry), with phosphatidylserine exposure at the cell surface, leads to transbilayer movement and consequent hydrolysis of SM at the cytoplasmic side of the plasma membrane [24]. Scrambling and loss of SM has profound effects on the physico-chemical properties, e.g. cholesterol content and fluidity [24,27], of the plasma membrane (microdomains), which facilitates membrane blebbing, vesicle shedding and apoptotic body formation. Replenishment of SM to the apoptotic cell prevents these surface changes. Thus, the breakdown of SM, rather than the production of Cer, seems to be the determining factor for these morphological changes in the apoptotic cell [24] (Figure 1).

While Cer is formed, in the effector phase of apoptosis, at the cytoplasmic side of the plasma membrane from the bulk SM that was initially located in the outer leaflet [24], there is also evidence for a stimulus-induced [e.g. by tumour necrosis factor α (TNFα) or vitamin D3] nSMase activity acting on the minor SM pool (10–20%) in the inner leaflet of the plasma membrane [28,29]. Whether this pool topologically corresponds to rafts/caveolae, and whether the conversion of SM into Cer at these sites (at 15–60 min) is relevant for apoptosis and may have a signalling or a membrane structural function, remains to be seen.

**Topology of ceramide formation: an important variable**

In an attempt to modulate Cer accumulation by boosting its glycosylation, we overexpressed glucosylceramide synthase (GCS) retrovirally in Jurkat cells [30]. Such an approach was previously undertaken by Cabot and co-workers [31,32], and appeared to be effective in the attenuation of the Cer response of MCF-7 cells to various chemotherapeutic drugs as well as TNFα. Moreover, it protected cells against the cytotoxicity of these stimuli. We found that stable GCS overexpression in Jurkat cells caused a 10-fold increase in GCS activity and a 7-fold elevated basal glucosylceramide level in vitro, as expected. However, Cer that accumulated during apoptosis induced via CD95 or by exposure to etoposide, γ-radiation or bacterial SMase, was not glycosylated by GCS, nor was apoptosis itself affected [30]. Similar results were obtained in GM95 melanoma cells stably transfected with functional GCS, and
exposed to various drugs (Veldman and Levade, submitted for publication). Therefore, GCS located in the Golgi is topologically segregated from Cer produced from SM in the plasma membrane. In contrast, de novo synthesized Cer, as well as an exogenously supplied cell-permeable C2-Cer analogue (inducing apoptosis), were efficiently glycosylated [30], apparently owing to different Cer topology and different physico-chemical behaviour of the short-chain Cer species, respectively. This is just one illustration that exogenous Cer analogues often do not mimic the behaviour of endogenously produced Cer, and one should be cautious to use it as a tool in a cell biological setting (e.g. as argument that Cer would be a second messenger) [5,23].

It is clear from these findings that only de novo synthesized Cer molecules (and cell-permeable Cer analogues) have access to GCS, and that Cer formed in the plasma membrane does not spontaneously transfer to other, intracellular membranes (as noted already above [5,15]). Thus, if the de novo pathway is responsible for Cer generation in the MCF-7 cells used by Cabot and co-workers [33], this may well explain why GCS expression attenuates Cer in that system but not in Jurkat and GM95 cells. That GCS protects against apoptosis induction in the MCF-7 cells is more difficult to explain. Since effects on lipid levels and viability in this cell system were only seen after a very long time (days) of exposure to the cytotoxic drug, adriamycin, or to TNFα [31,32], they may have been secondary to general alterations in cellular lipid homeostasis. In any case, we note here that although GCS expression and activity may somehow protect certain cancer cells (e.g. breast) from apoptosis, this may not be the case in other cell types [30].

**Alkyl-lysophospholipids as modulators of signal transduction and membrane lipid turnover/composition, and as inducers of apoptosis**

Since SM in the plasma membrane is important for its physico-chemical properties, microdomain formation, and blebbing and vesiculation during apoptosis, we sought to modulate membrane lipid composition by interfering with phospholipid biosynthesis and to look for effects on apoptosis induction. For this, we used the synthetic alkyl-lysophospholipid, 1-O-octadecyl-2-O-methyl-glycerol-3-phosphocholine (ALP; Et-18-OCH3; Edelfosine), an anticancer agent that induces apoptosis in a variety of tumour cells [34-37]. ALP incorporates easily into cell membranes [38] with pleiotropic effects on signal transduction. ALP interferes with phospholipid metabolism and with subsequent formation of lipid second messengers that act in mitogenic and cell survival pathways [36]. It inhibits the anti-apoptotic mitogen-activated protein kinase/extracellular-signal-regulated protein kinase and phosphoinositide 3-kinase/protein kinase B (PKB) pathways, and stimulates the pro-apoptotic stress-activated protein kinase/c-Jun N-terminal kinase pathway.

**Figure 2**

Effect of ALP and lysoPC on apoptosis and 14C incorporation into PC in S49 and S49AR cells

Exogenous lysoPC (25 μM) is rapidly (within 15 min) acylated to PC, thus preventing ALP-induced apoptosis in S49 cells. ALP (Et-18-OCH3; 15 μM) inhibits [14C]choline incorporation into PC (de novo pathway: measured at 4 h) with consequent apoptosis induction (measured after 5 h) in S49 but not in S49AR cells. The alternative (compensatory) [14C]lysoPC into PC pathway is not affected by ALP.
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In this way, ALP strongly enhances radiation-induced apoptosis in several tumour cell lines [35,36].

Perhaps the most important physiological target of ALP is CTP:cholinephosphate cytidylyltransferase [39], the enzyme that converts phosphocholine into CDP-choline in the endoplasmic reticulum, being the rate-limiting step in phosphatidylcholine (PC) biosynthesis. Using a mouse T-lymphoma cell line, S49, we found that ALP strongly inhibits PC synthesis and that the ensuing apoptosis can be prevented by providing the cells with an alternative pathway with which to synthesize PC, that is the acylation of exogenously supplied lysoPC (Figure 2), in agreement with Jackowski’s work [37]. Thus, continuous PC synthesis is important for cell survival/proliferation, while insufficient synthesis of new PC somehow generates a stress signal that tells the cell to turn on its apoptotic death machinery [37,40].

Do sphingolipids in rafts determine sensitivity of cells to stress-induced apoptosis?

An ALP-resistant variant cell, S49AR, no longer undergoes ALP-induced apoptosis and, in addition, appeared to be cross-resistant to various other stress agents/treatments [41]. These cells no longer internalized (endocytosed) ALP (3H-labelled ET-18-OCH\text{3}. Interestingly, PC synthesis in these cell was normal (also in the presence of ALP), but synthesis of SM was blocked (Figure 3). This must have consequences for the physico-chemical properties of the plasma membrane [27], particularly lipid raft formation. Could defective SM synthesis and raft assembly prevent ALP internalization and confer resistance to apoptosis? Indeed, artificial disruption of lipid rafts in S49 cells, e.g. by cholesterol depletion with β-cyclodextrin [42], inhibited ALP uptake and the induction of apoptosis (data not shown). We hypothesize that intact membrane rafts are ‘sensors’ for ALP and perhaps other stress agents that need membrane endocytosis to be effective, and that cells can escape from these toxic agents by shutting off their endocytosis through a block in SM synthesis and functional raft formation.

Concluding remarks

We have emphasized here the membrane structural and topological significance of Cer and SM in the induction and execution phases of apoptosis. For that matter, the glycosphingolipids may possibly have a similar significance. A second messenger function for Cer in the classical sense, like for diacylglycerol, remains possible [7] but more rigorous proof is needed. It should be realized that, if Cer is tightly packed in a membrane microdomain (raft/caveolus), and given its very small polar headgroup, its interaction with a...
possible cytoplasmic target protein is less likely. If it still does, it may rather do so outside the raft in a more fluid (loosely packed) environment, that may allow penetration of a protein domain into the lipid bilayer. This remains possible because rafts are not stable and static structures, but dynamic structures where molecules can move laterally in and out, albeit in a restricted fashion [42,43]. Membrane ‘sidedness’ further adds to this complexity. Perhaps, in addition to the structural role for Cer and SM in the outer leaflet of the plasma membrane, there may be a more dynamic, signalling role in the inner leaflet, as actually suggested [28,29]. In this regard, we need to know the precise composition of the individual halves of the raft bilayer, how they interact with each other, and out, albeit in a restricted fashion [42,43].

References

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