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Role of Ceramide 1-Phosphate in the Regulation of Cell Survival and Inflammation

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1. Introduction

Cell and tissue homeostasis is essential for normal development of an organism. When this is altered, metabolic dysfunctions and disease are prone to occur. Therefore, the maintenance of an appropriate balance in the activation / inhibition of the different metabolic pathways and cell signaling systems is simply vital.

Many lipids, including simple sphingolipids, are known to regulate cell activation and metabolism (Gomez-Munoz et al., 1992; Gomez-Munoz, 1998; Gomez-Munoz, 2004; Gomez-Munoz, 2006; Hannun & Obeid, 2008; Chen et al., 2011; Hannun & Obeid, 2011) . Some of them, including sphingosine, ceramides and their phosphorylated forms, sphingosine 1-phosphate (S1P) and ceramide 1-phosphate (C1P) have been described as crucial regulators of key processes that are essential for normal development, and have also been involved in the establishment and progression of different diseases (Gangoiti et al., 2008a; Arana et al., 2010). In particular, ceramides can induce cell growth arrest and cause apoptosis, when they are generated (Hannun et al., 1986; Kolesnick, 1987; Kolesnick & Hemer, 1990; Merrill & Jones, 1990; Merrill, 1991; Hannun, 1994; Kolesnick & Golde, 1994; Hannun & Obeid, 1995; Hannun, 1996; Spiegel & Merrill, 1996; Merrill et al., 1997; Kolesnick et al., 2000; Hannun & Obeid, 2002; Merrill, 2002). Nonetheless, although in general, ceramides are negative signals for cell survival, in neurons they can induce cell growth (Goodman & Mattson, 1996; Ping & Barrett, 1998; Brann et al., 1999; Song & Posse de Chaves, 2003; Plummer et al., 2005). Also, ceramides play important roles in the regulation of cell differentiation, inflammation, tumor development (Okazaki et al., 1990; Mathias et al., 1991; Dressler et al., 1992; Hannun, 1994; Kolesnick & Golde, 1994; Hannun & Obeid, 1995; Gomez-Munoz, 1998; Menaldino et al., 2003), bacterial and viral infections, and ischemia-reperfusion injury(Gulbins & Kolesnick, 2003). More recently, ceramides have been associated with insulin resistance through activation of protein phosphatase 2A and the subsequent dephosphorylation and inactivation of protein kinase B (PKB) (Schmitz-Peiffer, 2002; Adams et al., 2004; Stratford et al., 2004), and toll-like receptor 4 (TLR4)-dependent induction of inflammatory cytokines, a fact essential for TLR4-dependent insulin resistance (Holland et al., 2011).

Concerning ceramide generation, there are three different mechanisms by which these molecules can be synthesized in cells. Ceramides can be generated by i) *de novo* synthesis,

which takes place in the endoplasmic reticulum (ER), ii) by the action of different sphingomyelinases (SMases) in the plasma membrane, lysosomes, or mitochondria, and iii) by reacylation of sphingosine, a pathway known as the salvage or recycled pathway (Hannun & Obeid, 2011). The biosynthetic and degradative pathways of ceramide are shown in figure 1, where further products of ceramide metabolism are also indicated.

Natural ceramides tipically have long *N*-acyl chains ranging from 16 to 26 carbons in length (Merrill, 2002; Pettus et al., 2003a; Merrill et al., 2005), and some times longer in tissues such as skin. Many studies have used a short-chain analog (N-acetylsphingosine, or C_2-ceramide) in experiments with cells in culture because it can be incorporated into cells more easily and rapidly than long-chain ceramides. Of note, although C_2-ceramide was suggested not to occur *in vivo*, recent studies demonstrated that C_2-ceramide does exist in mammalian tissues. In particular, C_2-ceramide was found in rat liver cells (Merrill et al., 2001; Van Overloop et al., 2007), and brain tissue (Van Overloop et al., 2007). Ceramide generation is also relevant because this sphingolipid is the precursor of important bioactive molecules that can also regulate cellular functions. For instance, stimulation of ceramidases results in generation of sphingosine (Fig. 1), which was first described as a physiological inhibitor of protein kinase C (PKC) (Hannun et al., 1986). There are numerous reports in the scientific literature showing that PKC is inhibited by exogenous addition of sphingosine to cells in culture. Moreover, Merrill and co-workers demonstrated that addition of the ceramide synthase inhibitor fumonisin B1 to J774.A1 macrophages to increase the levels of endogenous sphingoid bases, also inhibited protein kinase C (Smith et al., 1997). Further work showed that sphingosine can affect the activity of other important enzymes that are involved in the regulation of metabolic or cell signaling pathways such as the Mg^{2+} dependent form of phosphatidate phosphohydrolase (Jamal et al., 1991; Gomez-Munoz et al., 1992), phospholipase D (PLD) (Natarajan et al., 1994), or diacylglycerol kinase (DAGK) (Sakane et al., 1989; Yamada et al., 1993). Sphingosine, in turn, can be phosphorylated by the action of sphingosine kinases to generate S1P, which is a potent mitogenic agent and can also inhibit apoptosis in many cell types (Olivera & Spiegel, 1993; Wu et al., 1995; Spiegel et al., 1996; Spiegel & Merrill, 1996; Spiegel & Milstien, 2002; Spiegel & Milstien, 2003). More recently, we demonstrated that S1P stimulates cortisol (Rabano et al., 2003) and aldosterone secretion (Brizuela et al., 2006) in cells of the zona fasciculata or zona glomerulosa, respectively, of bovine adrenal glands, suggesting that S1P plays an important role in the regulation of steroidogenesis.

A major metabolite of ceramide in cells is ceramide-1-phosphate (C1P), which is formed directly through phosphorylation of ceramide by the action of ceramide kinase (CerK) (Fig. 1). There is increasing evidence suggesting that C1P can regulate cell proliferation and apoptosis (Reviewed in (Gomez-Munoz, 1998; Gomez-Munoz, 2004)), and Chalfant and co-workers have implicated C1P in inflammatory responses (Reviewed in (Chalfant & Spiegel, 2005; Lamour & Chalfant, 2005)). In addition, Shayman's group demonstrated that C1P plays a key role in phagocytosis (Hinkovska-Galcheva & Shayman; Hinkovska-Galcheva et al., 1998; Hinkovska-Galcheva et al., 2005).

The aim of the present chapter is to review and update recent progress on the regulation of cell survival and inflammation by C1P.

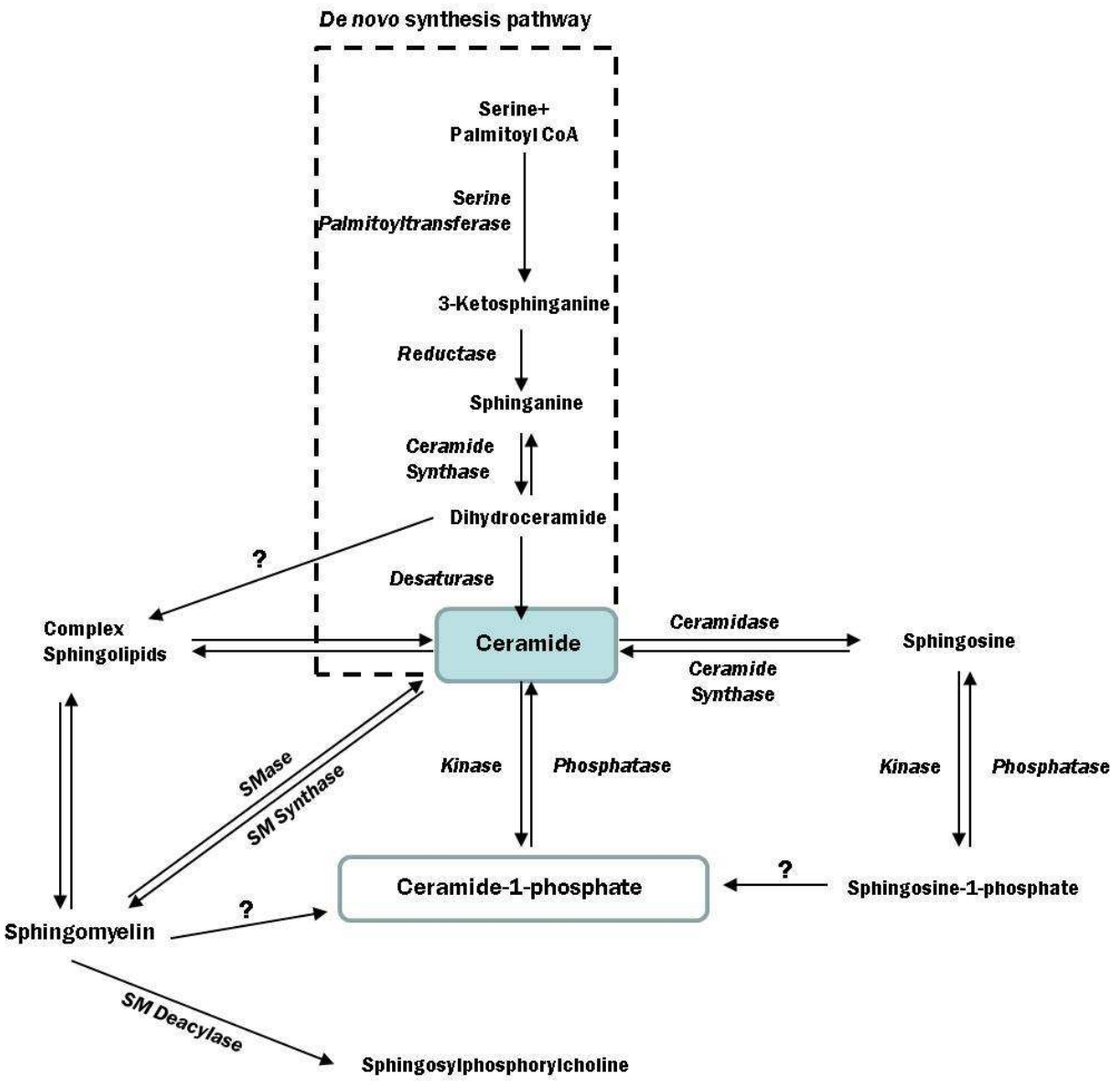


Fig. 1. Biosynthesis of simple sphingolipids in mammalian cells. Ceramide is the central core of sphingolipid metabolism. It can be produced by *de novo* synthesis through the concerted action of serine palmitoyltransferase and dihydroceramide synthase or by degradation of sphingomyelin (SM) through sphingomyelinase (SMase) activation. Ceramides can also be generated through metabolism of more complex sphingolipids. Phosphorylation of ceramide by ceramide kinase gives rise to ceramide-1-phosphate. The reverse reaction is catalyzed by ceramide-1-phosphate phosphatase, or by lipid phosphate phosphatases. Alternatively, ceramide can be degraded by ceramidases to form sphingosine, which can, in turn, be phosphorylated to sphingosine-1-phosphate by sphingosine kinases. The reverse reaction is catalyzed by sphingosine-1-phosphate phosphatases, or by lipid phosphate phosphatases. Sphingosine-1-phosphate lyase breaks down Sphingosine-1-phosphate to hexadecenal and ethanolamine phosphate, both of which can be recycled back to generate phosphatidylethanolamine. Sphingomyelin *N*-deacylase generates sphingosylphosphorylcholine, also known as lysosphingomyelin.

2. Biosynthesis of ceramide 1-phosphate. The essential role of ceramide kinase

At present, the only enzyme known to produce C1P in mammalian cells is ceramide kinase (CerK). This enzyme was first observed in brain synaptic vesicles (Bajjalieh et al., 1989), and was later found in human leukemia HL-60 cells (Kolesnick & Hemer, 1990). Cerk was first reported to be confined to the microsomal membrane fraction, but more recent studies indicate that it is mainly located in the cytosol (Mitsutake et al., 2004). These contradictory observations may arise from the different degrees of enzyme expression in different cell types, and it may also be possible that subcellular localization of this enzyme varies depending on cell metabolism. In this connection, Van Veldhoven and co-workers found that tagged forms of human CerK (FLAG-HsCerK and EGFP-HsCerK fusions), upon expression in Chinese Hamster Ovary (CHO) cells, were mainly localized to the plasma membrane, whereas no evidence for association with the ER was observed (Van Overloop et al., 2006). These findings are in agreement with those of Boath et al. (Boath et al., 2008) who showed that ceramides are not phosphorylated at the ER but must be transported to the Golgi apparatus for phosphorylation by CerK. When C1P is synthesized, it traffics from the Golgi network along the secretory pathway to the plasma membrane, where it can be back-exchanged into the extracellular environment and then bind to acceptor proteins such as albumin or lipoproteins (Boath et al., 2008). These observations are consistent with published work by Chalfant's group (Lamour et al., 2007), and it was demonstrated that CerK utilizes ceramide transported to the trans-Golgi apparatus by ceramide transport protein (CERT). In fact, downregulation of CERT by RNA interference resulted in strong inhibition of newly synthesized C1P, suggesting that CERT plays a critical role in C1P formation. However, Boat et al (Boath et al., 2008) reported that the transport of ceramides to the vicinity of CerK is not dependent upon CERT intervention. The reason for such discrepancy is unknown at the present time, but it is possible that the different experimental approaches used in those studies rendered different results. Specifically, whilst Lamour and co-workers used siRNA technology to inhibit CERT (Lamour et al., 2007), Boath and co-workers utilized pharmacological inhibitors (Boath et al., 2008). Also, it might be possible that different cell types may have different subcellular distribution of CerK, and / or that expression of this enzyme activity is not the same in all cell types.

With regards to the regulation of CerK, its ability to move intracellularly from one compartment to another and the dependency on cations (mainly Ca^{2+} ions) for activity seem to be well established. More recently, CerK has been proposed to be regulated by phosphorylation/dephosphorylation processes (Baumruker et al., 2005), and that it can be myristoylated at its N-terminus, a feature that is related to targeting proteins to membranes. Nonetheless, cleavage of the myristoylated moiety did not affect the intracellular localization of the enzyme. In addition, both CerK location and activity seem to require the integrity of its PH domain, which actually includes the myristoylation site, as deletion of this domain abolishes both the specific subcellular localization of the enzyme, as well as its activity (Baumruker et al., 2005).

Although CerK is thought to be the only enzyme for production of C1P, it was reported that bone marrow-derived macrophages (BMDM) from CerK-null mice (CerK-/-) still had significant levels of C1P (Boath et al., 2008). This observation suggests that there are other metabolic pathways, at least in mammals, capable of generating C1P independently of CerK.

Specifically, formation of C_{16}-C1P, which is a major species of C1P in cells, was not abolished in CerK-/- BMDM. Two alternative pathways for generation of C1P in cells might be: i) acylation of S1P by a putative acyl transferase that would catalyze the formation of a N-linked fatty acid in the S1P moiety to form C1P, and ii) cleavage of sphingomyelin (SM) by the action of a D-type SMase (SMase D), which would generate choline and C1P in an analogous manner to that of phospholipase D acting on phosphatidylcholine to produce choline and phosphatidic acid (PA). However, work from our own lab (Gomez-Munoz et al., 1995a) and that of others (Boath et al., 2008) demonstrated that acylation of S1P to form C1P does not occur in mammalian cells. Also, formation of C1P by the action of a putative SMase D has not yet been reported for mammalian cells. SMase D is a major component of the venom of a variety of arthropods including spiders of the gender *Loxosceles* (the brown recluse spider), such as *L. reclusa*. SMase D is also present in the toxins of some bacteria including *Corynebacterium pseudotuberculosis*, or *Vibrio damsela* (Truett & King, 1993). The bites of this spider result in strong inflammatory responses and may lead to renal failure, and occasionally lead to death (Lee & Lynch, 2005). Although we found no evidence for an analogous activity of SMase D in rat fibroblasts (Gomez-Munoz et al., 1995a), this possibility should be explored in more detail using different types of cells; so it is possible that SMase D may still be the cause for C1P generation in selective tissues.

Concerning regulation, mammalian CerK was demonstrated to be highly dependent on Ca^{2+} ions for activity (Van Overloop et al., 2006). More recently, it has been shown that treatment of human lung adenocarcinoma A549 cells and Chinese hamster ovary cells (CHO) with orthovanadate, a potent inhibitor of tyrosine phosphatases, increased CerK expression potently (Tada et al., 2010), suggesting a possible regulation of CerK by phosphorylation/dephosphorylation processes on tyrosine residues. Also, it has been suggested that CerK expression can be regulated through activation of Toll-like receptor 4 (TLR-4) by agonists such as the bacterial toxin lipopolysaccharide (Rovina et al., 2010).

The cloning of CerK (Sugiura et al., 2002) opened a new avenue of research that led to determination of important structural properties of this enzyme. The protein sequence has 537 amino acids with two protein sequence motifs, an N-terminus pleckstrin homology (PH) domain, and a C-terminal region containing a Ca^{2+}/calmodulin binding domain. Using site-directed mutagenesis, it was found that leucine 10 in the PH domain is essential for the catalytic activity of CerK (Kim et al., 2005). In addition, it was reported that the interaction between the PH domain of CERK and phosphatidylinositol 4,5-bisphosphate regulates the plasma membrane targeting and the levels of C1P (Kim et al., 2006). CERK also contains the five conserved sequence stretches (C1-C5) that are specific for lipid kinases (Reviewed in (Baumruker et al., 2005)).

With regards to substrate specificity, it was reported that phosphorylation of ceramide by CERK is stereospecific (Wijesinghe et al., 2005). The latter report also showed that a minimum of a 12-carbon acyl chain was required for normal CERK activity, whereas the short-chain ceramide analogues C_8-ceramide, C_4-ceramide, or C_2-ceramide were poor substrates for CERK. It was concluded that CERK phosphorylates only the naturally occurring D-erythro-ceramides (Wijesinghe et al., 2005). However, C_2-ceramide has been shown to also be a good substrate for CerK, especially when albumin is used as a carrier, and that C_2-ceramide can be converted to C_2-C1P within cells (Van Overloop et al., 2007). This raises the possibility that C_2-C1P is also a natural sphingolipid, capable of eliciting

important biologic effects, as previously demonstrated (i.e. stimulation of cell proliferation (Gomez-Munoz et al., 1995a)). These observations suggested that substrate presentation is an important factor when testing CerK activity and that the use of different vehicles may result in different outcomes. Also, it should be borne in mind that CerK expression may not be the same in all cell types. The importance of CERK in cell signaling was emphasized in experiments using specific small interfering RNA (siRNA) to silence the gene encoding for CerK. Downregulation of CerK blocked the response of the enzyme to treatment with ATP, the calcium ionophore A23187, or interleukin 1-betta (Pettus et al., 2003b; Chalfant & Spiegel, 2005), and led to a potent inhibition of arachidonic acid release and PGE_2 formation in A549 lung adenocarcinoma cells. The relevance of CerK in cell biology was also highlighted in studies using CerK null mice; specifically, a potent reduction in the amount of neutrophils in the blood and spleen of these animals compared to their wild type counterparts was observed, whereas de amount of leukocytes, other than neutrophils, was increased in those mice. These observations suggested an important role of CerK in neutrophil biology (Graf et al., 2008). In addition to CerK, a ceramide kinase-like (CERKL) protein was identified in human retina (Tuson et al., 2004), and this was subsequently cloned (Bornancin et al., 2005). However, CERKL failed to phosphorylate ceramide or other related lipids, under conditions commonly used to measure CERK activity. Therefore, the role of this protein in cell biology is unclear at the present time.

CerK has also been reported to exist in dicotyledonous plants, where it was associated to the regulation of cell survival (Bi et al., 2011). Also, it has been recently found that a conserved cystein motif is critical for rice CerK activity and function (Bi et al., 2011). However, no reports on the possible existence of Cerk in monocot plants are available at the present time.

3. Catabolism of ceramide 1-phosphate

From the above discussion, it should be apparent that C1P is a bioactive metabolite, capable of altering cell metabolism rapidly and potently. So, the existence of enzymes capable of degrading C1P seemed to be feasible for regulation of C1P levels. The identification of a specific C1P phosphatase in rat brain (Shinghal et al., 1993), and hepatocytes (Boudker & Futerman, 1993), together with the existence of CerK suggested that ceramide and C1P are interconvertible in cells. C1P phosphatase is enriched in brain synaptosomes and liver plasma membrane fractions, and appeared to be distinct from PA phosphohydrolase, the phosphatase that hydrolyzes PA. Nonetheless, C1P can also be converted to ceramide by the action of a PA phosphohydrolase that is specifically located in the plasma membrane of cells (Waggoner et al., 1996). The latter enzyme belongs to a family of at least three mammalian lipid phosphate phosphatases (LPPs) (Brindley & Waggoner, 1998). LPPs have recently been shown to regulate cell survival by controlling the levels of intracellular PA and S1P pools (Long et al., 2005), and also to regulate leukocyte infiltration and airway inflammation (Zhao et al., 2005). Dephosphorylation of C1P might be a way of terminating its regulatory effects, although the resulting formation of ceramide could potentially be detrimental for cells. Controlling the levels of ceramide and C1P by the coordinated action of CERK and C1P phosphatases, may be of crucial importance for the metabolic or signaling pathways that are regulated by these two sphingolipids. It could be speculated that another possibility for degradation of C1P might be its deacylation to S1P, which could then be cleaved by lyase activity to render a fatty aldehyde and ethanolamine phosphate (Merrill & Jones, 1990), or to

sphingosine by the action of S1P phosphatases (Fig. 1). However, no C1P deacylases or lyases have so far been identified in mammalian tissues, suggesting that the only pathway for degradation of C1P in mammals is through phosphatase activity.

4. Ceramide 1-phosphate and the control of cell growth and death

The first report showing that C1P was biologically active was published in 1995 (Gomez-Munoz et al., 1995a). C1P was found to have mitogenic properties as it stimulated DNA synthesis and cell division in rat or mouse fibroblasts (Gomez-Munoz et al., 1995a; Gomez-Munoz et al., 1997). Subsequent studies using primary macrophages, demonstrated that like for most growth factors, the mechanisms whereby C1P exerted its mitogenic effects implicated stimulation of the mitogen-activated protein kinase kinase (MEK)/Extracellularly regulated kinases 1-2 (ERK1-2), phosphatidylinositol 3-kinase (PI3-K)/protein kinase B (PKB, also known as Akt), and c-Jun terminal kinase (JNK) pathways (Gangoiti et al., 2008b). In addition, C1P caused stimulation of the DNA binding activity of the transcription factor NF-κB, and the selective inhibitors of MEK, PI3-K, and JNK (PD98059, LY290042, and SP600125), respectively) completely blocked NF-κB activation. Another major target of PKB is glycogen synthase kinase-3β (GSK-3 β), which expression was increased in the presence of C1P. This led to up-regulation of cyclin D1, and c-Myc, two important markers of cell proliferation that are targets of GSK-3β.

In addition, we found that C1P-stimulated macrophage proliferation, involved activation of sphingomyelin synthase (SMS), an enzyme that catalyzes the transfer of phosphocholine from phosphatidylcholine (PC) to ceramide to synthesize sphingomyelin (SM). The other by-product of this reaction is diacylglycerol (DAG), which is a well-established activator of protein kinase C (PKC). Conventional and novel PKC isoforms respond to DAG by translocating to the plasma membrane so that these enzymes can then express their activity and act on signaling events. In this connection, C1P stimulated the translocation and activation of the alpha isoform of PKC (PKC-α) in macrophages, and this resulted to be essential for stimulation of cell growth by C1P (Gangoiti et al., 2010c).

In a more recent report, it has been demonstrated that another essential kinase involved in the regulation of cell proliferation by C1P is the mammalian target of rapamycin (mTOR) (Gangoiti et al., 2010a). Activation of this kinase was tested my measuring the phosphorylation state of its downstream target p70S6K after treatment with C1P. Activation of mTOR/ p70S6K was dependent upon prior activation of PI3-K, as selective inhibition of this kinase blocked mTOR phosphorylation and activation. In addition, C1P caused phosphorylation of PRAS40, a component of the mTOR complex 1 (mTORC1) that is absent in mTORC2, and inhibition of the small G protein Ras homolog enriched in brain (Rheb), which is also a specific component of mTORC1, completely blocked C1P-stimulated mTOR phosphorylation, DNA synthesis and macrophage growth. C1P also caused phosphorylation of another Ras homolog gene family member, RhoA, and inhibition of its downstream effector RhoA-associated kinase (ROCK) also blocked C1P-stimulated mTOR and cell proliferation. It was concluded that mTORC1, and RhoA/ROCK are essential components of the mechanism whereby C1P stimulates macrophage proliferation. However, phospholipase D (PLD), and cAMP are not involved in the mitogenic effect of C1P (Gomez-Munoz et al., 1995a; Gomez-Munoz et al., 1997). Concerning intracellular calcium levels, which have also been implicated in the regulation of cell proliferation, the situation is controversial.

Although short-chain C1Ps failed to induce Ca^{2+} mobilization in fibroblasts (Gomez-Munoz et al., 1995a; Gomez-Munoz et al., 1997) or neutrophils (Rile et al., 2003), and natural C_{16}-C1P did not alter intracellular Ca^{2+} concentrations in A549 cells (Pettus et al., 2004), C_2-C1P- or C_8-C1P, caused intracellular Ca^{2+} mobilization in calf pulmonary artery endothelial (CAPE) cells (Gijsbers et al., 1999), thyroid FRTL-5 (Hogback et al., 2003), or Jurkat T-cells (Colina et al., 2005), suggesting that regulation of Ca^{2+} homeostasis may be cell type specific.

Finally, it should be pointed out that C1P has been recently shown to be a key mediator in the development and survival of retina photoreceptors, and to also play a critical role in photoreceptor differentiation (Miranda et al., 2011)

Apart from its mitogenic effect, another mechanism by which C1P controls cell homeostasis is by prevention of apoptosis (reviewed in (Gangoiti et al., 2010b)). We previously demonstrated that natural C1P blocked apoptosis in bone marrow-derived macrophages (Gomez-Munoz et al., 2004; Gomez-Munoz et al., 2005), and this was confirmed by Mitra and co-workers (Mitra et al., 2007) who found that down-regulation of CerK in mammalian cells reduced growth, and promoted apoptosis. Also, downregulation of CerK blocked epithelial growth factor-induced cell proliferation. However, in contrast to these observations, it was reported that addition of the cell-permeable C_2-ceramide to cells overexpressing CerK led to C_2-C1P formation and stimulation of apoptosis (Graf et al., 2007). This controversy can be explained by the fact that overexpression of CerK would substantially increase the intracellular levels of C1P, especially when cells are supplied with high concentrations of exogenous cell permeable C_2-ceramide; this action would cause overproduction of C_2-C1P inside the cells, which is toxic at high concentrations (Gomez-Munoz et al., 1995a; Gomez-Munoz et al., 2004).

When cells become apoptotic, their metabolism undergoes important changes from early stages. For example, apoptotic bone marrow-derived macrophages express high acid sphingomyelinase (A-SMase) activity and show high levels of ceramides compared to non-apoptotic cells (Gomez-Munoz et al., 2003; Hundal et al., 2003). Of interest, inhibition of A-SMase activation resulted to be one of the mechanisms by which C1P blocks apoptosis (Gomez-Munoz et al., 2004). C1P also blocked the activity of A-SMase in cell-free systems (in vitro), suggesting that inhibition of this enzyme takes place by direct physical interaction of C1P with the enzyme.

Recent work by our group (Granado et al., 2009a) showed that ceramide levels are also increased in alveolar NR8383 macrophages when they become apoptotic. However, A-SMase activity was only slightly enhanced in these cells under apoptotic conditions. This suggested the intervention of a different pathway for ceramide generation in these cells. In subsequent work we demonstrated that the mechanism whereby ceramide levels increased in apoptotic alveolar macrophages involved activation of serine palmitoyltransferase (SPT), the key regulatory enzyme of the de novo pathway of ceramide synthesis. Like for A-SMase, inhibition of SPT activation by treatment with C1P prevented the alveolar macrophages from entering apoptosis. These findings led to conclude that C1P promotes macrophage survival by blocking ceramide accumulation, and action that can be brought about through inhibition of either A-SMase activity, or SPT, depending on cell type.

The prosurvival effect of C1P was highlighted by the demonstration that intracellular levels of C1P were substantially decreased when the cells became apoptotic. It was hypothesized

that depletion of intracellular C1P could result in the release of A-SMase from inhibition, thereby triggering ceramide generation an apoptotic cell death (Gomez-Munoz et al., 2004). Once generated, ceramides act on different intracellular targets to induce apoptosis. One of these targets is protein kinase B (or Akt), a kinase that lies downstream of PI3-K, a major signaling pathway through which growth factors promote cell survival. Using two different experimental approaches, it was demonstrated that PI3-K was also a target of C1P (Gomez-Munoz et al., 2005). On one hand, PI3-K activation was demonstrated by immunoprecipitation of the enzyme from whole cell lysates and assayed in vitro using ^{32}P-phosphatidylinositol. On the other hand, an in vivo approach provided evidence of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) formation in intact cells that were prelabeled with ^{32}P-orthophosphate (Gomez-Munoz et al., 2005). PIP3 is a major product of PI3-K, and was shown to directly inhibit A-SMase (Testai et al., 2004). Therefore, it could be speculated that PI3-K activation might potentiate the inhibitory effect of C1P on A-SMase through generation of PIP3. C1P stimulated the phosphorylation of PKB, which was sensitive to inhibition by wortmannin or LY294002, thereby confirming that PI3-K was the enzyme responsible for its phosphorylation. These two PI3-K inhibitors also blocked the prosurvival effect of C1P, as expected (Gomez-Munoz et al., 2005). Another relevant finding was that C1P caused IkB phosphorylation and stimulation of the DNA binding activity of NF-kB in primary cultures of mouse macrophages (Gomez-Munoz et al., 2005). Of note, C1P up-regulated the expression of anti-apoptotic Bcl-XL, which is a downstream target of NF-kB. The latter results provided the first evidence for a novel biological role of natural C1P in the regulation of cell survival by the PI3-K/PKB/NF-kB pathway in mammalian cells (Gomez-Munoz et al., 2005).

As mentioned above, C1P can be metabolized to ceramide by different phosphatases, and then further converted to sphingosine and S1P by the coordinated actions of ceramidases and sphingosine kinases. Therefore, it could be speculated that the effects of C1P might be mediated through C1P-derived metabolites. However, usually ceramides and C1P exert opposing effects, (i.e. on PLD activation, adenylyl cyclase inhibition, or Ca^{2+} mobilization), and C1P is not able to reproduce the effects of S1P (Gomez-Munoz et al., 1995a; Gomez-Munoz et al., 1995b; Gomez-Munoz et al., 1997; Gomez-Munoz, 1998). Also, ceramides can decrease the expression of Bcl-XL (Chalfant & Spiegel, 2005), whereas C1P causes its up-regulation (Gomez-Munoz et al., 2005). Finally, no ceramidases capable of converting C1P into S1P have so far been reported to exist in mammalian cells, and S1P and C1P inhibit A-SMase through different mechanisms (Gomez-Munoz et al., 2003; Gomez-Munoz et al., 2004). Therefore, it can be concluded that C1P acts on its own right to regulate cell homeostasis. The above observations suggest that regulation of the enzyme activities involved in ceramide and C1P metabolism is essential for cell fate. Elucidation of the mechanisms controlling ceramide and C1P levels may help develop new molecular strategies for preventing metabolic disorders, or designing novel therapeutic agents for treatment of disease.

5. Ceramide 1-phosphate and the control of inflammation

Inflammation is, in principle, a beneficial process for protecting the organism against infection or injury. However, it can be detrimental when it becomes out of control. Apart from the classical signaling pathways and metabolites that are involved in the regulation of

inflammation, it is now well accepted that ceramides are key elements in the inflammatory response (Lamour & Chalfant, 2005; Wijesinghe et al., 2008; Gomez-Munoz et al., 2010). For instance, it was reported that activation of A-SMase and the subsequent formation of ceramides play an important role in pulmonary infections as it facilitates internalization of bacteria into lung epithelial cells (Gulbins & Kolesnick, 2003). In this context, inhibition of A-SMase by C1P could be important to reduce or prevent infection in the lung.

Inflammatory mediators include chemokines, cytokines, vasoactive amines, products of proteolytic cascades, phospholipases, or lipids such as eicosanoids and sphingolipids. A major mediator of inflammation is PLA_2 activity. In particular, group IV cytosolic $cPLA_2$ (or $cPLA_2$-alpha) has been involved in receptor-dependent and independent production of eicosanoids, which are major components of inflammatory responses. Sphingolipids, including ceramides, have also been described as key mediators of inflammation (Hayakawa et al., 1996; Serhan et al., 1996; Manna & Aggarwal, 1998; Newton et al., 2000). More recently a role for ceramide in the development of allergic asthmatic responses and airway inflammation was established (Masini et al., 2008), and exogenous addition of C_2-ceramide to cultured astrocytes induced 12-lipoxigenase leading to generation of reactive oxygen species (ROS) and inflammation (Prasad et al., 2008). Also, A-SMase-derived ceramide was involved in platelet activating factor (PAF)-mediated pulmonary edema (Goggel et al., 2004). Subsequently, it was proposed that at least some of the pro-inflammatory effects of ceramides might in fact be mediated by its further metabolite C1P. The first report on the regulation of arachidonic acid (AA) release and the production of prostaglandins by C1P was from the laboratory of Charles Chalfant (Pettus et al., 2003b). This group demonstrated that C1P was able to stimulate AA release and prostanoid synthesis in A549 lung adenocarcinoma cells. In a follow up report, the same group showed that the mechanism whereby C1P stimulates AA release occurs through direct activation of $cPLA_2$ (Pettus et al., 2004). Subsequently, it was found that C1P is a positive allosteric activator of $cPLA_2$-alpha, and that it enhances the interaction of the enzyme with PC (Subramanian et al., 2005). In further work, the same group demonstrated that activation of $cPLA_2$-alpha by C1P is chain length-specific; in particular, C1P bearing acyl chains equal or higher than six carbons were able to efficiently activate $cPLA_2$-alpha in vitro, whereas shorter acyl chains (in particular C_2-C1P) were unable to activate the enzyme. It was concluded that the biological activity of C_2-C1P does not occur via eicosanoid synthesis (Wijesinghe et al., 2008). Also, C1P was shown to act in coordination with S1P to ensure maximal production of prostaglandins. Specifically, S1P was shown to induce cyclooxigenase-2 (COX-2) activity, which then uses $cPLA_2$-derived AA as substrate to synthesize prostaglandins (Pettus et al., 2005). Further details on the role of C1P in inflammatory response can be found in different reviews (Chalfant & Spiegel, 2005; Lamour et al., 2007; Wijesinghe et al., 2007), Wijesinghe et al., and recent work by Murayama and co-workers (Nakamura et al., 2011).

6. Ceramide 1-phosphate and the control of cell migration

Macrophage populations in tissues are determined by the rates of recruitment of monocytes from the bloodstream into the tissue, the rates of macrophage proliferation and apoptosis, and the rate of macrophage migration or efflux. Recently, our group demonstrated that exogenous addition of C1P to cultured Raw 264.7 macrophages stimulated cell migration

(Granado et al., 2009b). Interestingly, this action could only be observed when C1P was applied to the cells exogenously, and not by increasing the intracellular levels of C1P (i.e. through agonist stimulation of CerK, or by using the ''caging" strategy to deliver C1P intracellularly (Lankalapalli et al., 2009)). This observation led us to identify a specific receptor through which C1P stimulates chemotaxis. This putative receptor seems to be located in the plasma membrane, has low affinity for C1P and has an apparent Kd of approximately 7.8 μM. The receptor is specific for C1P and is coupled to Gi proteins. Ligation of this receptor with C1P caused phosphorylation of ERK1–2, and PKB, and inhibition of either of these pathways completely abolished C1P-stimulated macrophage migration. Moreover, C1P stimulated the DNA binding activity of NF-kB, and blockade of this transcription factor resulted in full inhibition of macrophage migration. These observations suggest that MEK/ERK1-2, PI3-K/PKB (or Akt) and NF-kB are crucial signaling pathways for regulation of cell migration by C1P. It was concluded that this newly identified receptor could be an important drug target for treatment of illnesses in which cell migration is a major cause of pathology, as it occurs in atherosclerosis or in the metastasis of tumors.

7. Other relevant biological actions of C1P

In a previous report, Hinkovska-Galcheva et al (Hinkovska-Galcheva et al., 1998) showed that endogenous C1P can be generated during the phagocytosis of antibody-coated erythrocytes in human neutrophils that were primed with formylmethionylleucylphenylalanine. More recently, the same group demonstrated that C1P is a key mediator of neuthophil phagocytosis (Hinkovska-Galcheva et al., 2005). In addition, it was reported that C1P can be formed in neutrophils upon incubation with cell-permeable [^{3}H]*N*-hexanoylsphingosine (C_6-ceramide) (Rile et al., 2003), and Riboni and co-workers (Riboni et al., 2002) found that C1P can be generated in cerebellar granule cells both from SM-derived ceramide and through the recycling of sphingosine produced by ganglioside catabolism. C1P can be also generated by the action of interleukin 1-betta on A549 lung adenocarcinoma cells (Pettus et al., 2003b), or by stimulation of bone marrow-derived macrophages with macrophage-colony stimulating factor (M-CSF) (Gangoiti et al., 2008b). We found that C1P is present in normal bone marrow-derived macrophages isolated from healthy mice (Gomez-Munoz et al., 2004), and that C1P levels are substantially decreased in apoptotic macrophages. These observations are consistent with recent findings showing that CerK plays a key role in the stimulation of cell proliferation in A549 human lung adenocarcinoma cells (Mitra et al., 2007), and the induction of neointimal formation via cell proliferation and cell cycle progression in vascular smooth muscle cells by C1P (Kim et al., 2011).

8. Conclusion

The implication of simple sphingolipids in the regulation of cell activation and metabolism has acquired special relevance in the last two decades. Most attention was first paid to the effects elicited by ceramide because this sphingolipid turned out to be essential in the regulation of cell death, differentiation, senescence, and various metabolic disorders and diseases. However, its phosphorylated form, C1P, was thought not to be so important. However, C1P has emerged as a crucial bioactive sphingolipid, and this chapter highlights the relevance of C1P in cell biology. Specifically, C1P has now been established

as key regulator of cell growth and survival, and its relevance in the regulation of cell migration is beginning to emerge. Also importantly, the discovery that C1P can act both intracellularly or as receptor ligand opens a broad avenue to investigate its implication in controlling cell metabolism. In addition to this, C1P has been postulated to be a potent proinflammatory agent, acting directly on $cPLA_2$ to trigger eicosanoid production. Therefore, C1P and CerK, the major enzyme responsible for its biosynthesis, may be key targets for developing new pharmacological strategies for treatment of illnesses associated to cell growth and death, and cell migration , such as chronic inflammation, cardiovascular diseases, neurodegeneration, or cancer.

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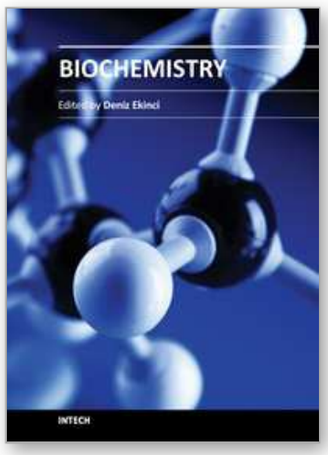
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Over the recent years, biochemistry has become responsible for explaining living processes such that many scientists in the life sciences from agronomy to medicine are engaged in biochemical research. This book contains an overview focusing on the research area of proteins, enzymes, cellular mechanisms and chemical compounds used in relevant approaches. The book deals with basic issues and some of the recent developments in biochemistry. Particular emphasis is devoted to both theoretical and experimental aspect of modern biochemistry. The primary target audience for the book includes students, researchers, biologists, chemists, chemical engineers and professionals who are interested in biochemistry, molecular biology and associated areas. The book is written by international scientists with expertise in protein biochemistry, enzymology, molecular biology and genetics many of which are active in biochemical and biomedical research. We hope that the book will enhance the knowledge of scientists in the complexities of some biochemical approaches; it will stimulate both professionals and students to dedicate part of their future research in understanding relevant mechanisms and applications of biochemistry.

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