Published in final edited form as:

Cell Signal. 2013 April ; 25(4): 786–795. doi:10.1016/j.cellsig.2013.01.009.

Ceramide 1-phosphate stimulates glucose uptake in macrophages

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Abstract

It is well established that ceramide 1-phosphate (C1P) is mitogenic and antiapoptotic, and that it is implicated in the regulation of macrophage migration. These activities require high energy levels to be available in cells. Macrophages obtain most of their energy from glucose. In this work, we demonstrate that C1P enhances glucose uptake in RAW264.7 macrophages. The major glucose transporter involved in this action was found to be GLUT 3, as determined by measuring its translocation from the cytosol to the plasma membrane. C1P-stimulated glucose uptake was blocked by selective inhibitors of phosphatidylinositol 3-kinase (PI3K) or Akt, also known as protein kinase B (PKB), and by specific siRNAs to silence the genes encoding for these kinases. C1P-stimulated glucose uptake was also inhibited by pertussis toxin (PTX) and by the siRNA that inhibited GLUT 3 expression. C1P increased the affinity of the glucose transporter for its substrate, and enhanced glucose metabolism to produce ATP. The latter action was also inhibited by PI3K- and Akt-selective inhibitors, PTX, or by specific siRNAs to inhibit GLUT 3 expression.

Keywords

Ceramide 1-phosphate; Ceramides; Glucose uptake; Macrophages; Sphingolipids; Sphingosine 1-phosphate

1. Introduction

Ceramide 1-phosphate (C1P) is now recognized to be an important regulator of cell homeostasis. First, we discovered that C1P was a potent stimulator of DNA synthesis and cell division in rat fibroblasts [1] and macrophages [2]. Subsequent studies led us to demonstrate that C1P also promoted cell survival [3-5]. The initially identified mechanisms involved in C1P-stimulated cell proliferation included activation of the phosphatidylinositol 3-kinase (PI3K)/Akt (also known as protein kinase B) pathway, as well as stimulation of extracellularly regulated kinases 1 and 2 (ERK1/2), and c-Jun N-terminal kinase (JNK) [2]. More recently, we showed that C1P activates protein kinase C alpha (PKCα) [6], and the mammalian target of rapamycin (mTOR) [7], which are also important kinases implicated in the regulation of cell proliferation [8]. Concerning cell survival, we showed that C1P inhibited apoptosis, an action that involved blockade of acid sphingomyelinase [5] or serine palmitoyltransferase [9] activities, depending on cell type. Another important observation

was that C1P stimulated cell motility. In this connection, we found that stimulation of macrophage migration required the interaction of exogenous C1P with cell membranes, while elevation of the intracellular levels of C1P did not enhance cell motility. These observations led us to identify a specific site on cell membranes (possibly a receptor) that seems to be essential for regulation of macrophage migration by C1P [10]. Other groups have reported on the control of inflammatory responses [11-13], the stimulation of phagocytosis [14,15], or neutrophil degranulation [16] by C1P.

Cell growth and survival require energy derived from glucose, which is the major energy source for mammalian cells [17], and also from amino acids [18]. Glucose and amino acids control protein synthesis by regulating translation factors such as eukaryotic initiation factor (eIF) 2B and eIF4F [19,20], and the relative contributions of these metabolic fuels to macrophage energy requirements depend on their availability [21]. In whole animals, removal of glucose from the circulation involves stimulation of glucose transport into metabolic tissues, and glucose homeostasis is maintained predominantly by insulin. Although an adequate supply of glucose is crucial for basal cell metabolism and thus for maintaining cell growth and viability, the regulation of glucose transport into cells in systems other than those involving insulin is poorly understood. Stress induced by a variety of agents activates glucose transport by stimulating the translocation of transporters from intracellular sites to the plasma membrane [22], or by increasing transporter expression [23]. In addition to insulin, various other hormones and bioactive molecules are capable of stimulating glucose uptake [24-29]. For example, activation of α 1-adrenergic or endothelin A receptors results in enhanced glucose uptake independent of insulin levels. Moreover, it is well established that many lipids, including some phospholipids, are important cell signaling molecules, some of which can also stimulate glucose uptake. In particular, lysophosphatidylcholine (LPC), which is involved in diverse biological activities including cell proliferation, tumor cell invasiveness, and inflammation [30,31], has recently been shown to stimulate glucose uptake in adipocytes [32]. Therefore, the control of glucose levels is not a simple event that can be entirely attributable to insulin, but rather is a complex process that implicates a variety of physiological and pathological regulators.

The present work was undertaken to examine whether C1P could stimulate glucose uptake and metabolism in macrophages, and to define the mechanism(s) involved in this action.

2. Materials and methods

2.1. Materials

N-Hexadecanoyl-D-erythro-sphingosine-1-phosphate (C16:0-Ceramide 1-phosphate) (C1P) was supplied by Matreya. [^3H]-2-Deoxy-D-glucose ([^3H]2-DG, specific activity, 20 Ci/mmol), and [γ - ^{32}P]ATP were purchased from Perkin Elmer. Culture medium, Dulbecco's Modified Eagle's Medium (DMEM) was from Lonza. LY 294002, PD 98059, pertussis toxin, Protease Inhibitor Cocktail, sphingomyelinase (from *Bacillus cereus*), dequalinium, JTE013, 4-[[4-(4-chlorophenyl)-2-thiazolyl]amino] phenol (SKI-II), and SP600125 were from Sigma-Aldrich. 10-DEBC was from Tocris. 2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol hydrochloride (FTY720) was from Cayman. Fetal bovine serum (FBS) was from Gibco. Ceramides, sphingosine 1-phosphate, VPC23019 and W146 were purchased from Avanti Polar Lipids. BHNB-C1P was synthesized as described previously [33]. Nitrocellulose membranes, protein markers, and BCA assay reagents were purchased from Bio-Rad. The ATP-Glo Kinase KitTM was from Promega. Antibodies to phospho-Akt, Akt, GAPDH, and the p85 subunit of PI3K were from Cell Signaling. Antibodies to Glut 3 and sphingosine kinase 1, and GLUT 3 siRNA were from Santa Cruz. PI3K and Akt siRNAs were from Ambion. All of the other chemicals and reagents were of the highest grade.

available. The transilluminator (Darkreader DR-45M) used to expose the cells to light was from Clare Chemical.

2.2. Cell culture

RAW264.7 macrophages and C2C12 myoblasts were purchased from the American Type Culture Collection (Manassas, VA, USA). RAW264.7 cells were cultured in high glucose DMEM supplemented with 10% heat-inactivated FBS, 50 mg/l of gentamicine, and 2 mM L-glutamine at 37 °C in a humidified atmosphere containing 5% CO₂. The C2C12 myoblasts were routinely grown in DMEM supplemented with 10% FBS, 50 mg/l of gentamicine and 2 mM L-glutamine. For most experiments, cells were seeded in 12- or 6-well dishes with 0.5 ml or 1 ml of culture medium, respectively, and used when ~40% confluent. For experiments on GLUT3 translocation cells were seeded in 60 mm plates.

2.3. Delivery of C1P to cells in culture

An aqueous dispersion (in the form of liposomes) of C1P was added to cultured macrophages, as reported previously [2,4,5]. Stock solutions were prepared by sonicating C1P (1.66 mg) in sterile nanopure water (1 ml) on ice using a probe sonicator until a clear dispersion was obtained. The final concentration of C1P in the stock solution was approximately 2.6 mM. This procedure is considered preferable to using dispersions prepared by adding C1P in organic solvents because droplet formation is minimized and exposure of cells to organic solvents such as dodecane is avoided. We also delivered C1P to cells by using the photolabile caged C1P analog, 4-bromo-5-hydroxy-2-nitrobenzhydryl (BHNB)-C1P [33], which was dissolved in ethanol at 1.62 mM in the dark. The final ethanol concentration was <0.16%. The cells were exposed to 400–500 nm light in a transilluminator equipped with a 9 W lamp for 60 min at a distance of 1.5 cm at 37 °C, so as to release the C1P into the cytosol.

2.4. Measurement of glucose uptake

Glucose uptake was measured by the zero-trans method using [³H]2-DG as described previously [34]. Briefly, RAW264.7 cells were washed in Krebs-Ringer phosphate (KRP) buffer, resuspended at 1×10⁶ cells/well in 12-well dishes, and allowed to adhere for 2 h at 37 °C. Cells were treated with or without C1P at various concentrations for different time periods. Cells were then washed twice with phosphate-buffered saline (PBS) and preincubated at 37 °C for 5 min in glucose-free DMEM without FBS. Glucose (100 μM) and [³H]2-DG (1 μCi/well) were then added and glucose uptake was determined at 5 min under conditions in which the uptake was linear for 10 min [34]. Glucose uptake was stopped by addition of 500 μl of ice-cold KRP buffer with 0.3 mM phloretin for 5 min. Then, the cells were washed twice in KRP buffer and collected in 500 μl of 0.3 mM NaOH 1% (v/v) SDS, and the radioactivity was determined by liquid-scintillation counting. When inhibitors were used, the cells were preincubated with the inhibitors for 30 min before the addition of C1P.

2.5. Assay of sphingosine kinase activity

Sphingosine kinase activity was determined as described previously with minor modifications [35]. Briefly, RAW264.7 macrophages were seeded in 60-mm dishes at 2×10⁶ cells/well and incubated in DMEM containing 10% FBS. Two hours later, the medium was removed and the cells were washed twice with PBS. After the cells were incubated for 24 h with 500 ng/ml of LPS in DMEM without FBS [36]. Cells were washed with cold PBS and harvested in SK1 buffer (containing 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 15 mM NaF, 1 mM β-mercaptoethanol, 40 mM β-glycerophosphate, 0.4 mM PMSF, 10% glycerol, 0.5% Triton X-100, and Protease Inhibitor Cocktail (Sigma)). After brief sonication and determination of protein concentration using BCA protein assay, 100 μg of protein were

incubated in 100 μ l of reaction mixture containing sphingosine (50 μ M, delivered in 4 mg/ml fatty acid-free bovine serum albumin), [γ - 33 P] ATP (5 μ Ci, 1 mM dissolved in 10 mM MgCl_2) with or without 4-[[4-(4-chlorophenyl)-2-thiazolyl]amino] phenol (SKI-II) inhibitor at 20 or 40 μ M, and SK1 buffer for 30 min at 37 $^{\circ}$ C. The reaction was terminated by the addition of 10 μ l of 1 M HCl and 400 μ l of chloroform/methanol/HCl (100:200:1, v/v/v) and allowed to stand at room temperature for 10 min. Subsequently, 120 μ l of chloroform and 120 μ l of 2 M KCl were added, and samples were centrifuged at 3000 $\times g$ for 5 min. Two hundred microliters of the organic phase were transferred to new glass tubes and dried under nitrogen stream. Samples were re-suspended in chloroform/methanol/HCl (100:100:1, v/v/v). Lipids were then resolved on silica thin layer chromatography plates using 1-butanol/methanol/acetic acid/water (8:2:1:2, v/v/v/v) as a solvent system and visualized by autoradiography. The radioactive spots corresponding to S1P were scraped from the plates and the radioactivity was determined by liquid-scintillation counting. Background values were determined in negative controls in which sphingosine was not added to the reaction mixture.

2.6. Assay of glycolysis

Glycolysis was measured using the ATP Kinase-Glo commercial assay kit (Promega Corp.). Macrophages were washed in KRP buffer, resuspended at 1×10^6 cells/well, in 12-well dishes and allowed to adhere for 2 h at 37 $^{\circ}$ C. Cells were treated with or without C1P (15 μ M) for different time periods. When inhibitors were used, the cells were preincubated with the inhibitors for 30 min before addition of C1P. The cells were washed twice with PBS, harvested, and lysed in ice-cold homogenization buffer. Then, 50 μ l of each sample was placed in 96-well plates, with 50 μ l of the kinase-Glo reagent. Luminescence was measured using a Synergy HT (Biotek) plate reader equipped with Gen 5 software.

2.7. Treatment of cells with siRNA

RAW264.7 macrophages were seeded in 6-well dishes at 5×10^5 cells/well and incubated in DMEM containing 10% FBS. Four hours later, the medium was removed and the cells were washed twice with PBS. After the cells were incubated for 24 h with 800 μ l of Opti-MEM without antibiotics, 20 pmol siRNA in 200 μ l was added according to the manufacturer's instructions. Cells were then incubated for 5 h in culture medium containing 1 ml of Opti-MEM and 20% FBS. The next day, the cells were washed with PBS and the medium was replaced by fresh DMEM supplemented with 10% FBS. After 24 h of incubation, the medium was removed and the macrophages were washed twice with PBS. The cells were then preincubated with DMEM without FBS for 2 h, as indicated [34,37], and C1P or inhibitors were added as required.

2.8. Western blotting

Macrophages were harvested and lysed in ice-cold homogenization buffer as described [38]. Protein (20–40 μ g) from each sample was loaded and separated by SDS-PAGE, using 12% separating gels. Proteins were transferred onto nitrocellulose paper and blocked for 1 h with 5% skim milk in Tris-buffered saline (TBS) containing 0.01% NaN_3 and 0.1% Tween 20, pH 7.6, and then incubated overnight with the primary antibody in TBS/0.1% Tween at 4 $^{\circ}$ C. After three washes with TBS/0.1% Tween 20, the nitrocellulose membranes were incubated with horseradish peroxidase-conjugated secondary antibody at 1:4000 dilution for 1 h. Bands were visualized by enhanced chemiluminescence.

2.9. Preparation of crude membranes

RAW264.7 cells were harvested and lysed with a Dounce homogenizer in ice-cold homogenization buffer containing 1 μ l/ml of Protease Inhibitor Cocktail (Sigma-Aldrich) as

described [38], and the remaining intact cells and nuclei were removed by centrifugation at $500 \times g$ for 5 min at 4 °C. Cell membranes were pelleted by centrifugation at $100,000 \times g$ for 30 min at 4 °C and resuspended in the homogenization buffer. The samples were analyzed by Western blotting, as indicated above.

2.10. Statistical analyses

Results are expressed as means $\pm$ SEM of three independent experiments performed in triplicate, unless indicated otherwise. Statistical analyses were performed using the two-tailed, paired Student's *t*-test, where $p < 0.05$ was considered to be significant (GraphPad Prism software, San Diego, CA).

3. Results

3.1. C1P stimulates glucose uptake in macrophages

To examine whether C1P could stimulate glucose uptake we used [^3H] 2-DG to label the substrate, as indicated in [34]. RAW264.7 macrophages were incubated in the presence of various concentrations of C1P for different time periods. C1P stimulated glucose uptake in a concentration- and time-dependent manner (Fig. 1). We observed that C1P had a significant effect on glucose uptake at a concentration of 5 μM , with a maximal effect at 15 μM . Interestingly, this concentration of C1P was also optimum for inhibition of apoptosis in alveolar macrophages [9]. However, similar concentrations of ceramides did not stimulate glucose uptake in the macrophages (Fig. 2), in agreement with other studies [39]. Also, treatment of the macrophages with exogenous bacterial sphingomyelinase at concentrations known to generate relatively high amounts of ceramides at the plasma membrane (200–500 mU/ml) and inhibit cell growth [40] or phospholipase D [41,42] did not stimulate glucose uptake.

3.2. C1P-stimulated glucose uptake is mediated by the glucose transporter GLUT 3

Previous work showed that the major glucose transporter expressed in RAW264.7 macrophages is GLUT 3 [34]. We have confirmed this observation by using different specific antisera against various GLUT transporters, including GLUT 1, GLUT 3, GLUT 4, and GLUT 6, which are all expressed in RAW264.7 macrophages (data not shown). Therefore, we hypothesized that GLUT 3 was the glucose transporter responsible for the incorporation of glucose into macrophages after treatment with C1P. This possibility was evaluated using specific siRNA to inhibit GLUT 3 expression in the macrophages. Fig. 3A shows that silencing of the gene encoding for GLUT 3 with specific GLUT 3 siRNA completely blocked C1P-stimulated glucose uptake, thus demonstrating that GLUT 3 is the glucose transporter responsible for C1P-stimulated glucose uptake. Western-blotting revealed significant knockdown of GLUT 3 with 20 pmol/ml GLUT 3-targeted siRNA ($55.2 \pm 2.3\%$ inhibition, $n=3$) compared to non-targeted (Ctrl) siRNA (Fig. 3B, C).

It is known that activation of GLUT 3 requires its translocation from the cytosol to the plasma membrane of cells [43]. To assess whether C1P could stimulate GLUT 3 translocation, the protein levels of this glucose transporter were measured in the cytosolic and membrane fractions obtained from macrophages in the basal state, or after stimulation with C1P (15 μM). C1P induced a significant increase in the protein levels of GLUT 3 in the membrane fraction, which was concomitant with a decrease of this transporter in the cytosolic fraction (Fig. 4A, B). These results suggest that C1P stimulates GLUT 3 translocation, which is consistent with the time course for glucose uptake shown in Fig. 1, and with the inhibition of C1P-stimulated glucose uptake by GLUT 3 siRNA (Fig. 3). We also observed that insulin, which is a major regulator of glucose transport and metabolism and which functions primarily through stimulation of GLUT 4, did not affect glucose uptake

by the macrophages. However, as expected, insulin stimulated glucose uptake in myoblasts (Fig. 5). Moreover, kinetic analysis revealed that stimulation of glucose uptake by C1P was consistent with a 50% increase in transporter affinity for glucose. In particular, the K_m value of glucose uptake in control cells was decreased from 1.4 ± 0.2 mM to 0.7 ± 0.1 mM in C1P-treated cells, without significantly changing the V_{max} value. This indicates that C1P increases the affinity of the glucose transporter in the macrophages (Fig. 6).

3.3. C1P-stimulated glucose uptake requires activation of the PI3K/Akt pathway

In a previous study we found that C1P stimulated the PI3K/Akt and mitogen-activated protein kinase kinase (MEK)/ERK1/2 pathways in RAW264.7 macrophages, and that these effects were associated with the stimulation of cell migration by C1P [10]. Fig. 7A shows that inhibition of the PI3K/Akt pathway with LY294002, a selective inhibitor of PI3K, or with 10-DEBC, a selective inhibitor of Akt, completely blocked C1P-stimulated glucose uptake in these cells. Furthermore, siRNA silencing of the genes encoding for PI3K or Akt completely blocked C1P-stimulated glucose uptake (Fig. 7B). Western-blotting demonstrated a significant knockdown of PI3K ($46.9 \pm 7\%$ inhibition, $n=3$) (Fig. 7C, D) or Akt (38.8 ± 5.9 , $n=3$) (Fig. 7E, F) with 20 pmol/ml PI3K-targeted or Akt-targeted siRNA, respectively. However, inhibition of other kinases that are also stimulated by C1P, including ERK 1/2, c-Jun N-terminal kinase (JNK), or PKC α with the optimal inhibitory concentrations of PD98059, SP600125, or dequalinium, respectively [10], did not affect C1P-stimulated glucose uptake (Fig. 7G).

We previously reported that C1P can act intracellularly to stimulate cell growth [7,33] or inhibit apoptosis [4,5], and that it can bind to a putative Gi protein (PTX-sensitive)-coupled receptor to stimulate cell migration [10]. Therefore, studies were aimed at defining whether C1P-stimulated glucose uptake was dependent on or independent of receptor interaction. As shown in Fig. 8A, relatively low concentrations of PTX ($0.1 \mu\text{g/ml}$) completely blocked C1P-stimulated glucose uptake. In addition, PTX ($0.1 \mu\text{g/ml}$) completely blocked C1P-stimulated Akt phosphorylation (Fig. 8B, C), as well as the occurrence of GLUT 3 in the plasma membrane (Fig. 8D, E), actions that are all consistent with an involvement of the PI3K/Akt pathway, and activation of the glucose transporter GLUT 3 in this process. The observation that exogenous C1P acts through interaction with its putative receptor is supported by data obtained from experiments using the caged C1P analog, BHNb-C1P, which was previously found to be plasma membrane permeable and to bypass surface receptor(s) in macrophages [33]. The bioactive lipid C1P can be released into the cytosol upon photolysis using visible light that does not damage cellular components (wavelengths > 360 nm) [33]. Fig. 8F shows that contrary to exogenous C1P, intracellularly released C1P failed to stimulate glucose uptake, whereas it was able to stimulate DNA synthesis and proliferation of these same cells, or primary macrophages at concentrations as low as $1\text{--}2.5 \mu\text{M}$, as we previously reported [33]. Therefore, the caging/uncaging strategy allowed us to conclude that only extracellular C1P, but not C1P that was generated inside the cells, is responsible for stimulation of glucose uptake in the macrophages.

It has been shown that sphingosine 1-phosphate (S1P), a sphingolipid that is closely related to C1P, stimulates glucose uptake in various cell types [44-46], and we found that it also enhances glucose uptake in RAW264.7 macrophages (Fig. 9A). Many of the effects of S1P are mediated through interaction with plasma membrane receptors of the endothelin differentiation gene family (EDG), which are coupled to G_i proteins. There are five S1P receptors (S1P $_{1-5}$), and S1P-stimulated glucose uptake was shown to be mediated through receptor interaction [47]. Therefore, it could be speculated that C1P might be able to interact with any S1P receptor present in these cells. In this connection, it should be pointed out that RAW264.7 macrophages only express the S1P $_1$ and S1P $_2$ receptors [47,48], with S1P $_2$ being negatively involved in cell migration [49]. Using the S1P $_1$ specific receptor antagonists

W146, and VPC 23019, which inhibits S1P₁ and S1P₃ receptors, we observed that the stimulatory effect of S1P on glucose uptake was completely abolished whereas C1P-stimulated glucose uptake was not significantly altered (Fig. 9B). In addition, the S1P₂ inhibitor JTE013 affected neither S1P- nor C1P-stimulated glucose uptake (Fig. 9B). We and others previously reported that C1P is slowly metabolized to ceramide, and that it acts on its own right to elicit its effects independently of conversion to other metabolites including S1P [1,50,51]. To confirm that S1P was not involved in the stimulation of glucose uptake by C1P, glucose uptake was measured in macrophages that were preincubated for 24 h with 2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol hydrochloride (FTY720), a sphingosine kinase (SK) inhibitor that is known to induce proteolysis of SK1 [52]. Fig. 10A shows that this inhibitor did not significantly affect C1P-stimulated glucose uptake at concentrations at which it caused downregulation of SK1 (Fig. 10B, C) thereby demonstrating that the effect of C1P is not dependent upon S1P formation. Western-blotting revealed significant inhibition of SK1 levels with FTY720 (52.1±4.7% inhibition at 5 µM FTY720, n=3, and 42±3.7% inhibition, n=3 at 10 µM FTY720) compared to control values (Fig. 10B). S1P can also be formed by SK2, so experiments were also performed in the presence of 4-[[4-(4-chlorophenyl)-2-thiazolyl]amino]phenol] (SKI-II), a dual inhibitor of SK1 and SK2 [53]. Like for FTY720, SKI-II did not affect C1P-stimulated glucose uptake (Fig. 10D) at concentrations at which it potentially decreased SK activity in vitro (SKI-II decreased SK activity by 55.8±5.7%, n=3, at 20 µM, and by 58±10.2% at 40 µM. Basal SK specific activity was 2.04±0.19 pmol/min/mg protein, n=3). As expected from the latter observations, neither of these inhibitors significantly affected C1P-stimulated Akt phosphorylation (Fig. 11A, B, E, F) nor GLUT 3 translocation to the plasma membrane (Fig. 11C, D, G, H). These findings support the notion that C1P acts through a specific receptor to stimulate glucose uptake, which is consistent with our previous work on C1P stimulation of cell migration [10].

3.4. C1P stimulates glucose metabolism

We next investigated whether C1P was able to enhance glucose metabolism to generate ATP. This was achieved by measuring the production of ATP after treatment of the macrophages with an optimum concentration of C1P (15 µM). Fig. 12 shows that C1P significantly increased ATP levels in a time-dependent manner (panel A). As shown in Fig. 7, PI3K/Akt is a major pathway involved in the regulation of glucose uptake by C1P. Therefore, we examined whether this pathway was also involved in the enhancement of ATP generation by C1P. Fig. 12B shows that inhibition of PI3K or Akt with the selective inhibitors LY294002 or 10-DEBC, respectively, completely blocked C1P-stimulated ATP formation. In addition, pretreatment of the macrophages with specific PI3K or Akt siRNAs completely blocked C1P-stimulated ATP generation (Fig. 12C), suggesting that the PI3K/Akt pathway is key to both stimulation of glucose uptake and enhancement of its metabolism to produce ATP.

4. Discussion

Our previous work demonstrated that C1P is a major regulator of macrophage growth and death (for a review, see [8]), and more recently we found that it is a potent stimulator of macrophage migration [10]. These vital biological functions require high energy levels to be accomplished, and it is known that the major source of metabolic energy in macrophages is glucose [54-56]. The data reported in this work demonstrate that C1P stimulates glucose uptake as well as ATP production in RAW264.7 macrophages. It is known that the passage of glucose across cell membranes is facilitated by the GLUT family of transporters; although RAW264.7 macrophages express GLUT 1, 3, 4, and 6, we observed that the predominant glucose transporter in these cells is GLUT 3, in agreement with previous work [34]. Of interest, we found that C1P promoted the translocation of GLUT 3 from the cytosol to the

membrane fraction, suggesting a role for this transporter in C1P-stimulated glucose uptake. The involvement of GLUT 3 in the stimulation of glucose uptake by C1P was determined by measuring the incorporation of glucose into macrophages in the presence of selective inhibitors of this transporter, and by pre-incubating the cells with specific siRNA to inhibit GLUT3 expression. These experimental approaches led us to conclude that GLUT3 is the major glucose transporter in C1P-stimulated glucose uptake in the macrophages. In addition, C1P significantly increased the affinity of GLUT 3 for the substrate, which is consistent with the enhancement of glucose uptake by C1P-treated macrophages.

Since C1P is known to activate a number of enzymes including various kinases, we studied the mechanistic aspects of C1P-stimulated glucose uptake. Stimulation of macrophages with C1P led to the rapid phosphorylation of ERK1/2, JNK, PKC α and Akt, which are all involved in the regulation of macrophage growth, survival, and migration [2,6,10]. Therefore, the pathways these kinases belong to are all, in principle, suitable candidates for the regulation of glucose uptake by C1P. In this connection, the data presented in this work demonstrate that the PI3K/Akt pathway is the major mechanism by which C1P stimulates glucose uptake, as specific inhibition of either PI3K or Akt completely blocked this process. These findings are consistent with previous work in other cell types. For example, Rathmell et al. [57] found that Akt-directed glucose metabolism can promote growth-factor independent survival and that activated Akt promotes increased resting T-cell size, CD28-independent T-cell growth, and development of autoimmunity and lymphoma [58]. Furthermore, Ferreira et al. [59] reported that glucose uptake via GLUT3 in platelets is regulated by Akt, and Elstrom and co-workers [60] found that Akt stimulates aerobic glycolysis in cancer cells. Nonetheless, glucose uptake is regulated by different mechanisms other than activation of PI3K/Akt depending on the glucose transporter and cell type [17,61]. For example, lysophosphatidylcholine (LPC) does not require PI3K activation to stimulate glucose uptake in adipocytes, but is dependent upon activation of PKC- δ [32]. Furthermore, homocysteine sulfinic acid, a homologue of the amino acid cysteine, is a selective metabotropic glutamate receptor agonist that stimulates glucose uptake through the calcium-dependent AMPK-p38 MAPK-PKC- ζ pathway in skeletal muscle cells [62]. Also, carbachol (a non-hydrolyzable analog of acetylcholine) stimulates glucose uptake in skeletal muscle through stimulation of GLUT 4 translocation involving PKC- ϵ [63].

Another important aspect of the mechanism whereby C1P stimulates glucose uptake is the requirement of interaction of C1P with its putative plasma membrane receptor, independent of the presence of S1P receptors in these cells. In this regard, and in contrast to treatment with exogenous C1P, intracellular delivery of C1P (using the photolabile caged C1P analog BHNb-C1P, which traverses the plasma membrane in the caged form) did not enhance glucose uptake in the macrophages. Moreover, the results shown here indicate that the effect of C1P on stimulation of glucose uptake is independent of conversion to S1P, which is consistent with previous work [1,50,51].

The present study is the first report showing that C1P can stimulate glucose uptake and metabolism. It is concluded that this process requires the intervention of the GLUT 3 glucose transporter, and that activation of the PI3K/Akt pathway, possibly through interaction of C1P with its putative receptor [10], is a major mechanism by which C1P exerts this action.

Acknowledgments

This work was supported by grants BFU2009-13314/BFI from Ministerio de Ciencia e Innovación (MICINN) (Madrid, Spain), IT-705-13 from Departamento de Educación, Universidades e Investigación del Gobierno Vasco (GV/EJ, Spain), S-PE11UN017 and S-PE12UN040 from Departamento de Industria, Comercio y Turismo del Gobierno Vasco (Basque Government, GV/EJ, Spain) to AGM, and grant HL083187 from the National Institutes of

Health (USA) to RB. LA and AO are the recipients of fellowships from the Basque Government. I-G.R is the recipient of a fellowship from Ministerio de Ciencia e Innovación (MICINN) (Madrid, Spain), and MO is the recipient of a fellowship from the University of the Basque Country (GV/EJ, Spain). We are grateful to SGiker and UFI 11/20 (UPV/EHU) for technical support.

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Abbreviations

BHNB	4-bromo-5-hydroxy-2-nitrobenzhydryl
C1P	ceramide 1-phosphate
2-DG	2-Deoxy-D-glucose
ERK	extracellularly regulated kinases
FBS	fetal bovine serum
JNK	c-Jun N-terminal kinase
PBS	phosphate-buffered saline
PTX	pertussis toxin
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
S1P	sphingosine 1-phosphate
SMase	sphingomyelinase
SK	sphingosine kinase

Fig. 1.

C1P stimulates glucose uptake. A. RAW264.7 macrophages were stimulated with increasing concentrations of C1P for 1 h, as indicated. The cells were then washed and incubated with FBS- and glucose-free DMEM for 10 min. Glucose uptake was then determined as indicated in the Materials and methods section. B. Cells were treated as in panel A, except that they were challenged with C1P (15 μM) for different times, as indicated. Results are expressed relative to the control (Ctrl) value (panel A) or to the value at time 0 h (panel B) and are the mean $\pm$ SEM of 3 independent experiments performed in triplicate (* p <0.05; ** p <0.01).

Fig. 2.

Lack of effect of ceramides on glucose uptake in macrophages. RAW264.7 cells were seeded at 1×10^6 cells/35-mm dish. Macrophages were stimulated with C1P or with the cell permeable ceramides *N*-acetyl-D-sphingosine (C₂-ceramide, C₂-Cer) or *N*-hexanoyl-D-sphingosine (C₆-ceramide, C₆-Cer) at 15 μ M, or with bacterial sphingomyelinase (SMase) at 200 mU/ml and 500 mU/ml, as indicated. Glucose uptake was determined as mentioned in the Materials and methods section. Results are expressed relative to the control (Ctrl) value and are the mean $\pm$ SEM of 3 independent experiments (* $p < 0.05$).

Fig. 3.

Effect of GLUT 3 siRNA on C1P-mediated macrophage glucose uptake. A. Macrophages were seeded at 0.5×10^6 cells/35-mm dish and were preincubated for 5 h with specific GLUT 3 siRNA (20 pmol/ml) as indicated. The siRNA transfected cells were maintained in DMEM with 10% FBS for 48 h. They were then serum-starved for 2 h, and C1P (15 μ M) was added to the cells for 1 h. Glucose uptake was determined as indicated in the Materials and methods section. Results are expressed relative to the control (Ctrl) value and are the mean $\pm$ SEM of 4 independent experiments (** $p < 0.01$, control versus C1P-treated cells; ## $p < 0.01$, C1P-stimulated cells versus C1P-stimulated cells treated with GLUT 3 siRNA). B. The cells were seeded at 1×10^6 cells/60-mm dish, and then preincubated for 5 h with 20 pmol/ml of

control (Ctrl) siRNA or GLUT 3 siRNA, as indicated. Then cells were lysed by sonication and total GLUT 3 was analyzed by Western blotting using a specific antibody to GLUT 3, as described in the Materials and methods section. Equal loading of protein was monitored using a specific antibody to total GAPDH. Similar results were obtained in each of 3 replicate experiments. C. Results of scanning densitometry of the exposed film. Data are expressed as arbitrary units of intensity and are the mean $\pm$ SEM of three replicate experiments (**p<0.001, GLUT 3-treated cells versus Control siRNA-treated cells).

Fig. 4.

Effect of C1P on GLUT 3 translocation. Macrophages were seeded at 2×10^6 cells/60 mm dish in 2 ml of medium. The cells were lysed with a Dounce homogenizer and the membrane and soluble fractions were separated by ultracentrifugation as indicated in the Materials and methods section. The presence of GLUT 3 in the different cell fractions was analyzed by Western blotting using a specific antibody to GLUT 3. A. The results shown are from a representative experiment; similar data were obtained in each of three replicate experiments. Equal loading of protein was monitored using a specific antibody to p85 (membrane fraction) and GAPDH (cytosolic fraction). B. Results of scanning densitometry of exposed film. Data are expressed as arbitrary units of intensity relative to the value at time 0 h, and are the mean $\pm$ SEM of three replicate experiments (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Fig. 5.

Effect of insulin on glucose uptake in macrophages and myoblasts. RAW264.7 macrophages or C2C12 myoblasts were seeded in 6-well dishes at 1×10^6 cells/well. Cells were preincubated for 2 h in DMEM containing 5.5 mM glucose in the absence of FBS, and were stimulated with C1P (15 μ M) or insulin (100 nM) for 1 h, as indicated. Glucose uptake was determined as mentioned in the Materials and methods section. Results are expressed relative to the control (Ctrl) value and are the mean $\pm$ SEM of 4 independent experiments (* $p < 0.05$).

Fig. 6.

Effect of C1P on glucose transport kinetics. RAW264.7 cells were preincubated for 10 min in DMEM in the absence of glucose and FBS. The cells were then treated with increasing concentrations of glucose in the presence of [^3H]2-DG (1 $\mu\text{Ci}/\text{well}$) with or without C1P (15 μM) as indicated. Glucose uptake was determined as indicated in the Materials and methods section. Results are the mean $\pm$ SEM of 3 independent experiments.

Fig. 7.

Effect of PI3K and Akt inhibition on C1P-stimulated glucose uptake. Macrophages were seeded in 6-well dishes at 1×10^6 cells/well and incubated as indicated in Fig. 1. A. Cells were preincubated for 30 min with vehicle, 10 μ M LY294002 (a selective inhibitor of PI3K), or with 1 μ M 10-DEBC (a selective inhibitor of Akt) prior to treatment with C1P (15 μ M) for 1 h, as indicated. Results are expressed relative to the control (Ctrl) value and are the mean $\pm$ SEM of 3 independent experiments (* p < 0.05, control versus C1P-treated cells; # p < 0.05, C1P-treated cells versus C1P-treated cells in the presence of the corresponding inhibitor). (B) Macrophages were preincubated for 5 h with 20 pmol/ml of control (Ctrl) siRNA, PI3K siRNA, or Akt siRNA, as indicated. The siRNA transfected cells were maintained in DMEM containing 10% FBS for 48 h and were then serum-starved for 2 h. Then the cells were treated with C1P (15 μ M) for 1 h. Results are expressed relative to the control (Ctrl) value and are the mean $\pm$ SEM of 3 independent experiments (** p < 0.01, control versus C1P-treated cells; # p < 0.05, C1P-treated cells versus C1P-treated cells in the presence of the corresponding siRNA). C. Macrophages were seeded at 1×10^6 cells/60-mm dish, and were then preincubated for 5 h with 20 pmol/ml of control (Ctrl) siRNA or PI3K siRNA, as indicated. Then the cells were lysed by sonication and total PI3K was analyzed as described in the Materials and methods section. Equal loading of protein was monitored using a specific antibody to total GAPDH. Similar results were obtained in each of 3 replicate experiments. D. Results of scanning densitometry of the exposed film. Data are expressed as arbitrary units of intensity and are the mean $\pm$ SEM of three replicate experiments (** p < 0.01, PI3K siRNA-treated cells versus Control siRNA-treated cells). E. Cells were seeded as described in panel C, and were then preincubated for 5 h with 20 pmol/ml of control (Ctrl) siRNA or Akt siRNA, as indicated. Then macrophages were lysed by sonication and total Akt was analyzed by Western blotting as described in the Materials and

methods section. Equal loading of protein was monitored using a specific antibody to total GAPDH. Similar results were obtained in each of 3 replicate experiments. F. Results of scanning densitometry of the exposed film. Data are expressed as arbitrary units of intensity and are the mean $\pm$ SEM of three replicate experiments (* p <0.05, Akt siRNA-treated cells versus Control siRNA-treated cells). G. Cells were preincubated with vehicle (Ctrl), 10 μ M PD98059 (a selective inhibitor of mitogen-activated protein kinase kinase, which phosphorylates ERK), 1 μ M dequalinium (a selective inhibitor of PKC- α), or 1 μ M SP600125 (a selective inhibitor of JNK) for 30 min prior to treatment with 15 μ M C1P for 1 h. Results are expressed relative to the control (Ctrl) value and are the mean $\pm$ SEM of 3 independent experiments (* p <0.05, control versus C1P-treated cells; * p <0.05 or ** P <0.01, control versus C1P in the presence of the corresponding inhibitor).

Fig. 8.

Inhibition of C1P-stimulated glucose uptake by pertussis toxin (PTX). **A**. Cells were preincubated for 18 h with vehicle (Ctrl) or with 0.1 μ g/ml of PTX prior to treatment with C1P (15 μ M) for 1 h, as indicated. Data are expressed relative to the Ctrl value and are the mean $\pm$ SEM of three replicate experiments (* P <0.05, control versus C1P-treated cells, # p <0.01, C1P-treated cells versus C1P-treated cells in the presence of PTX). **B**. Cells were preincubated with or without PTX as in panel A, and were treated without (Ctrl) or with C1P (15 μ M) for 10 min, as indicated. Cells were lysed by sonication and Akt phosphorylation was analyzed by Western blotting using a specific antibody to phospho-Akt (p-Akt), as described in the Materials and methods section. Equal loading of protein was monitored using a specific antibody to total Akt. Similar results were obtained in each of 3 replicate experiments. **C**. Results of scanning densitometry of exposed film. Data are expressed as arbitrary units of intensity and are the mean $\pm$ SEM of three replicate experiments (** P <0.01, control versus C1P-treated cells, ## p <0.01 C1P-treated cells versus C1P-treated cells in the presence of PTX). **D**. Macrophages were preincubated with or

without PTX as in panel A, and were treated without (Ctrl) or with C1P (15 μ M) for 30 min. Then, the cells were lysed with a Dounce homogenizer and the membrane fraction was separated by ultracentrifugation as indicated in the Materials and methods section. The presence of GLUT 3 in the membrane fraction was analyzed by Western blotting using a specific antibody to GLUT 3, as described in the Materials and methods section. Equal loading of protein was monitored using a specific antibody to total p85. Similar results were obtained in each of 3 replicate experiments. E. Results of scanning densitometry of exposed film. Data are expressed as arbitrary units of intensity and are the mean $\pm$ SEM of three replicate experiments (**P<0.01, control versus C1P-treated cells, ##p<0.01, C1P-treated cells versus C1P-treated cells in the presence of PTX). F. Cells were treated with C1P (15 μ M) or with BHN-B-C1P (1 or 2.5 μ M) for 1 h, as indicated, and glucose uptake was measured as mentioned in the Materials and methods section. Results are expressed relative to control (Ctrl) value and are the means $\pm$ SEM of three independent experiments (*p<0.05).

Fig. 9.

The stimulation of glucose uptake by C1P is independent of interaction with S1P receptors. A. RAW264.7 macrophages were seeded in 6-well dishes at 1×10^6 cells/well, and incubated as in Fig. 1. They were then stimulated with increasing concentrations of S1P or with C1P (15 μM), as indicated. Data are expressed relative to the control (Ctrl) value and are the mean $\pm$ SEM of three replicate experiments (* $P < 0.05$, control versus C1P- or S1P-treated cells). B. Macrophages were seeded and incubated as indicated in Fig. 1. The cells were then preincubated for 30 min with 5 μM VPC23019 (a selective inhibitor of S1P₁ and S1P₃ receptors), or with 0.5 nM W146 (a specific inhibitor of S1P₁ receptor), or for 45 min with 1 μM JTE013 (a selective inhibitor of S1P₂) or vehicle prior to treatment with C1P (15 μM) or S1P (5 μM) for 1 h, as indicated. Glucose uptake was determined as mentioned in the Materials and methods section. Results are expressed relative to the control (Ctrl) value and are the mean $\pm$ SEM of 3 independent experiments (* $p < 0.05$ and ** $p < 0.01$, control versus C1P- or S1P-treated cells; ## $p < 0.05$ S1P-treated cells versus S1P-treated cells in the presence of W146 (0.5 nM); n.s., no significant).

Fig. 10.

Stimulation of glucose uptake by C1P is not affected by inhibitors of sphingosine kinase. RAW264.7 macrophages were seeded in 6-well dishes at 1×10^6 cells/well, and incubated as in Fig. 1. A. Macrophages were preincubated without (Ctrl) or with 2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol hydrochloride (FTY720, a selective inhibitor of sphingosine kinase 1, SK1) at the indicated concentrations for 24 h, prior to treatment with C1P (15 μ M), or vehicle (Ctrl) for 1 h. Glucose uptake was determined as indicated in the Materials and methods section. Results are expressed relative to the control (Ctrl) value and are the mean $\pm$ SEM of 3 independent experiments (* p < 0.05). B. Cells were preincubated with FTY720 as in panel A. Then, the cells were lysed by sonication and SK1 was analyzed by Western blotting using a specific antibody, as indicated in the Materials and methods section. Equal loading of protein was monitored using a specific antibody to total GAPDH. C. Results of scanning densitometry of exposed film. Data are expressed as arbitrary units of intensity relative to control (Ctrl) value and are the mean $\pm$ SEM of three replicate experiments (** P < 0.01). D. The cells were preincubated in the absence (Ctrl) or in the

presence of the SK inhibitor 4-[[4-(4-chlorophenyl)-2-thiazolyl]amino]phenol (SKI-II), at the indicated concentrations for 30 min prior to treatment with C1P (15 μ M), or vehicle (Ctrl) for 1 h. Glucose uptake was determined as indicated in the Materials and methods section. Results are expressed relative to the control (Ctrl) value and are the mean $\pm$ SEM of 4 independent experiments performed in triplicate (** p <0.01; *** p <0.001).

Fig. 11.

Sphingosine kinase inhibitors did not affect C1P-stimulated Akt phosphorylation or C1P-induced GLUT3 translocation. RAW264.7 macrophages were seeded in 6-well dishes at 1×10^6 cells/well, and incubated as in Fig. 1. A. Raw 264.7 macrophages were preincubated in the absence (Ctrl) or in the presence of FTY720 (5 μ M) for 24 h, and were then treated without (Ctrl) or with C1P (15 μ M) for 10 min. Then, the macrophages were lysed by sonication and Akt phosphorylation was analyzed by Western blotting using a specific antibody to phospho-Akt (p-Akt), as described in the Materials and methods section. Equal loading of protein was monitored using a specific antibody to total Akt. Similar results were obtained in each of 3 replicate experiments. B. Results of scanning densitometry of exposed film. Data are expressed as arbitrary units of intensity relative to each control and are the mean $\pm$ SEM of three replicate experiments (* P < 0.05). C. Macrophages were treated as in panel A. The presence of GLUT 3 in the membrane fraction was analyzed by Western

blotting using a specific antibody to GLUT 3, as described in the Materials and methods section. Equal loading of protein was monitored using a specific antibody to total p85. Similar results were obtained in each of 3 replicate experiments. D. Results of scanning densitometry of exposed film. Data are expressed as arbitrary units of intensity relative to the control (Ctrl) value and are the mean $\pm$ SEM of three replicate experiments (* p <0.05, control versus C1P-treated cells, *** P <0.001; FTY720-treated cells versus FTY720-treated cells with C1P). E. The macrophages were preincubated in the absence (Ctrl) or in the presence of SKI-II (20 μ M) for 30 min, and were then treated without (Ctrl) or with C1P (15 μ M) for 10 min, as indicated. Then, the macrophages were lysed by sonication and Akt phosphorylation was analyzed by Western blotting using a specific antibody to phospho-Akt (p-Akt), as described in the Materials and methods section. Equal loading of protein was monitored using a specific antibody to total Akt. Similar results were obtained in each of 3 replicate experiments. F. Results of scanning densitometry of exposed film. Data are expressed as arbitrary units of intensity relative to each control (Ctrl) and are the mean $\pm$ SEM of three replicate experiments (* P <0.05). G. Macrophages were treated as in panel E. The presence of GLUT 3 in the membrane fraction was analyzed by Western blotting using a specific antibody to GLUT 3, as described in the Materials and methods section. Equal loading of protein was monitored using a specific antibody to total p85. Similar results were obtained in each of 3 replicate experiments. H. Results of scanning densitometry of exposed film. Data are expressed as arbitrary units of intensity and are the mean $\pm$ SEM of three replicate experiments (* p <0.05, control versus C1P-treated cells; ** P <0.01, SKI-II-treated cells versus SKI-II-treated cells with C1P).

Fig. 12. C1P increases the intracellular levels of ATP. Involvement of the PI3K/Akt pathway. Macrophages were seeded in 6-well dishes at 1×10^6 cells/well, and incubated as in Fig. 1. A. The cells were stimulated with C1P (15 μ M) for different times, as indicated and ATP levels were determined using the ATP Kinase-Glo kit, as described in the Materials and methods section. B. Cells were preincubated with the PI3K inhibitor LY294002 (10 μ M), or with the Akt inhibitor 10-DEBC (1 μ M) for 30 min prior to treatment with vehicle or C1P (15 μ M) for 20 min, as indicated. C. Cells were preincubated for 5 h with 20 pmol/ml control (Ctrl) siRNA, or specific PI3K siRNA, or Akt siRNA, as indicated. The siRNA transfected cells were maintained in DMEM containing 10% FBS for 48 h. The cells were serum-starved for

2 h, and were then treated with C1P (15 μ M) for 20 min. Basal concentration of intracellular ATP was 4.2 ± 0.4 mM (mean $\pm$ SEM of nine independent experiments). Results are expressed relative to control (Ctrl) values and are the mean $\pm$ SEM of three independent experiments (* $p < 0.05$; ** $p < 0.01$, control versus C1P-treated cells; # $p < 0.05$, C1P-treated cells versus C1P-treated cells in the presence of the corresponding inhibitor or siRNA).