Hypoxia Enhances Sphingosine Kinase 2 Activity and Proves Sphingosine-1-Phosphate-Mediated Chemoresistance in A549 Lung Cancer Cells

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Abstract

Hypoxia and signaling via hypoxia-inducible factor-1 (HIF-1) is a key feature of solid tumors and is related to tumor progression as well as treatment failure. Although it is generally accepted that HIF-1 provokes tumor cell survival and induces chemoresistance under hypoxia, HIF-1-independent mechanisms operate as well. We present evidence that conditioned medium obtained from A549 cells, incubated for 24 h under hypoxia, protected naïve A549 cells from etoposide-induced cell death. Lipid extracts generated from hypoxia-conditioned medium still rescued cells from apoptosis induced by etoposide. Specifically, the bioactive lipid sphingosine-1-phosphate (S1P) not only was essential for cell viability of A549 cells but also protected cells from apoptosis. We noticed an increase in sphingosine kinase 2 (SphK2) protein level and enzymatic activity under hypoxia, which correlated with the release of S1P into the medium. Knockdown of SphK2 using specific small interfering RNA relieved chemoresistance of A549 cells under hypoxia and conditioned medium obtained from SphK2 knockdown cells was only partially protective. Coincubations of conditioned medium, with the further notion that p42/44 mitogen-activated protein kinase transmits autocrine survival signaling downstream of S1P1/3 receptors. Our data suggest that hypoxia activates SphK2 to promote the synthesis and release of S1P, which in turn binds to S1P1/3 receptors, thus activating p42/44 mitogen-activated protein kinase to convey autocrine or paracrine protection of A549 cells. (Mol Cancer Res 2009;7(3):393–401)

Introduction

Tissue hypoxia provokes a decrease in nutrient supply and is associated with pathologic conditions such as stroke, athero-
further clarification (15, 27, 28). Besides, S1P is exported from the cell by members of the ATP-binding cassette transporter family. Once secreted, S1P binds to one of its five G-protein-coupled receptors (S1P1-S1P5), activating downstream pathways such as p38 MAPK, p42/44 MAPK, PI3K, c-Jun NH2-terminal kinase, or Ca2+ signaling (29-31).

More recently, the role of lipids in cell survival under hypoxia/ischemia was discussed. Two reports showed activation of SphK1 and the release of S1P under hypoxia (32, 33). Anelli et al. identified HIF-2 as a positive regulator of SphK1 expression, whereas HIF-1 acted as a competitive inhibitor (33). In opposite, Schwall et al. suggested that both HIF-1 and HIF-2 act in concert to induce SphK1 protein under hypoxic conditions (32). New evidence suggests that SphK1 activity under hypoxia is mediated by reactive oxygen species (34). Irrespective of differences on mechanistic details, increased expression and activation of SphKs and subsequent formation of S1P has been correlated with enhanced survival (29), and an increased synthesis of S1P has been reported under hypoxic conditions (35). Additionally, N,N-dimethylsphingosine (DMS), an inhibitor of both SphK isoenzymes, was used to attenuate proliferation of smooth muscle cells under hypoxia (35). Moreover, mouse cardiac myocytes lacking functional SphK1 showed a reduced survival under hypoxia due to a diminished S1P release, which underscores the importance of SphK activity for cell survival under hypoxia (16).

Our work provides evidence that hypoxia caused an increase in SphK2 protein expression and activity, which provoked the formation and release of S1P. Extracellular S1P acted as a transferable survival factor, attenuating induction of apoptosis via binding to S1P1/S1P3 receptors and activation of p42/44 MAPK signaling in A549 cells.

**Results**

Previous work led to the identification of a chemoresistant phenotype in A549 lung carcinoma cells when subjected to hypoxia. Cells showed an abrogated caspase-3 activation and chromatin condensation, two hallmarks of apoptosis, in response to etoposide treatment (5). Stimulation of A549 cells with 150 μmol/L etoposide for 4 h under serum-free conditions provoked massive cell death followed by caspase-3 activity measurements (specific activity of caspase-3: normoxia, 2.1 ± 1.5 nmol/mg/min; normoxia + etoposide, 68.4 ± 18.0 nmol/mg/min). Induction of apoptosis was reduced by ~75%, when cells were preincubated for 24 h under hypoxia (specific activity of caspase-3: 16.4 ± 3.1 nmol/mg/min; Fig. 1A, black columns). To exclude drug-specific effects, we replaced etoposide and used staurosporine to induce apoptosis. Treatment of A549 cells with 1 μmol/L staurosporine caused significant caspase-3 activation, which could be prevented by preincubating cells under hypoxia for 24 h (Fig. 1A, white columns). To rule out that hypoxia-mediated protection from drug-induced cell death is cell type specific, we confirmed mechanisms of hypoxia-induced chemoresistance using human THP-1 macrophages. Treatment of THP-1 macrophages with 1 μmol/L staurosporine for 8 h under serum-free conditions caused caspase-3 activation (normalized to 100%), which was reduced by ~40% after preincubation of cells for 24 h under hypoxia (Fig. 1B).

In continuing experiments, we collected medium from A549 cells incubated for 24 h under normoxia [CM(normoxia)] or hypoxia [CM(hypoxia)] and stimulated naive cells with these conditioned media for 24 h under normoxic conditions. We noticed that CM(hypoxia) but not CM(normoxia) protected A549 cells from etoposide-induced cell death (Fig. 1C). To elucidate the nature of the transferable survival factor secreted under hypoxia, we first excluded proteins being involved by treating CM(hypoxia) with 50 μmol/L proteinase K (1 h, 37°C) followed by protein denaturation (2 h, 100°C). This treatment did not eradicate protection induced by CM(hypoxia) (Fig. 1C). Taking into account that lipid mediators are associated with cell protection, we extracted lipids from conditioned medium and tested for their cytoprotective potential. Lipid extracts from normoxia-conditioned medium [Lip-CM(normoxia)] did not protect cells from etoposide-induced cell death. However, lipid extracts from CM(hypoxia) [Lip-CM(hypoxia)] strongly reduced caspase-3 activity on etoposide treatment (Fig. 1C). These observations suggest that CM(hypoxia) contains a lipid molecule that provokes chemoresistance.

To identify the nature of the lipid present in CM(hypoxia), we screened several potential candidates described to interfere with apoptosis. Treatment with lysophosphatidic acid (10 μmol/L), platelet-activating factor (1 μmol/L), or ceramide-1-phosphate (1 μmol/L) for 24 h under normoxia failed to protect A549 cells from etoposide-induced cell death (Fig. 2A). Interestingly, S1P (10 μmol/L) reduced caspase-3 activity by ~35% compared with etoposide treatments (Fig. 2A). We further showed that 0.1 to 10 μmol/L S1P dose-dependently protected A549 cells from cell death induced by etoposide (Fig. 2B). To ascertain whether CM(hypoxia) or Lip-CM(hypoxia) contain S1P, we used 10 μmol/L DMS, an inhibitor of SphK isoenzymes, which is known to reduce cell viability at high concentrations, and coincubated DMS with S1P, Lip-CM(hypoxia), or CM(hypoxia). As expected, stimulation of cells with 10 μmol/L S1P for 24 h under normoxia caused cell death followed by caspase-3 activation (Fig. 2C). Induction of caspase-3 activity could be prevented by coincubating DMS with 10 μmol/L S1P, CM(hypoxia), or Lip-CM(hypoxia) (Fig. 2C). These results argue for a pivotal role of S1P in preserving cell viability and furthermore point to S1P being present in CM(hypoxia) or lipid extracts [Lip-CM(hypoxia)].

A prerequisite for S1P synthesis is the expression and/or activation of at least one SphK isoform under hypoxia. Because DMS is an inhibitor of both SphK isoenzymes, we went on to identify which isoform might be responsible for hypoxic protection. Regulation of SphK1 under hypoxia has been correlated to HIF activity, and due to the fact that the promoter of SphK2 also contains putative hypoxia response elements, we incubated cells under hypoxia or in the presence of the hypoxia-mimetic CoCl2 to test whether HIF transcription factors influence mRNA expression of SphKs. In A549 cells, both isoforms are expressed at a basal rate, but neither 100 μmol/L CoCl2 nor 4 h hypoxia caused changes in mRNA transcripts of SphK1 (Fig. 3A, white columns) or SphK2 (Fig. 3A, black columns). Interestingly, hypoxia (1-4 h) elevated the protein level of SphK2, whereas the protein amount of SphK1 was not induced (Fig. 3B). The expression of HIF-1α served as a marker for cellular hypoxia. Blocking protein synthesis with...
Cycloheximide pointed to protein expression being involved. Cycloheximide, shortly supplied before putting cells to 1% oxygen, blocked expression of SphK2 under hypoxia (Fig. 3C). Accompanied by increased protein expression of SphK2, we measured an increased SphK2 enzyme activity after 2 to 4 h of hypoxia (Fig. 3D, black columns), whereas SphK1 activity was not affected (Fig. 3D, white columns). To determine consequences of enhanced SphK2 activity, we next quantified extracellular concentrations of S1P in the supernatants of hypoxia-treated cells. There was an increase of extracellular S1P from 37 nmol/L at 4 h under normoxia compared with 145 nmol/L at 4 h of hypoxia (Fig. 3E).

We then asked whether enhanced SphK activity and the concomitant release of S1P contributed to protection of cells under hypoxia. Therefore, we incubated cells for 24 h under hypoxia in the presence of 5 μmol/L DMS and collected medium [CM(hypoxia/DMS)]. Quantification of extracellular S1P showed that inhibition of SphK activity in A549 cells attenuated the S1P release compared with cells that retain full SphK activity. CM(hypoxia) contained 354 nmol/L S1P versus 188 nmol/L found in CM(hypoxia/DMS). Accordingly, on treatment of A549 cells with etoposide, chemoresistance was attenuated in cells treated with CM(hypoxia/DMS) (Fig. 4A). This effect could be reversed by coincubating CM(hypoxia/DMS) with 1 μmol/L S1P (Fig. 4A). To answer the question whether SphK2 protein expression and its enzymatic activity caused chemoresistance under hypoxia, we used validated small interfering RNA (siRNA) against SphK2 to specifically inhibit SphK2 expression. We noted a reduction of SphK2 mRNA by ~50%, whereas transfection with either control siRNA (mock-siRNA) or SphK1-specific siRNA did not affect SphK2-mRNA transcripts (Fig. 4B). In A549 cells, with a knockdown of SphK2, hypoxia was less effective in protecting cells from etoposide-induced cell death when compared with naive cells. Transfection with mock-siRNA or SphK1-siRNA did not interfere with chemoresistance under hypoxia (Fig. 4C).
extending experiments, we checked whether the release of the survival molecule demands SphK2 expression. A549 cells were transfected with siRNA directed against SphK1, SphK2, or mock-siRNA followed by incubating transfected cells for 24 h under hypoxia to collected conditioned medium. Conditioned medium from cells with a knockdown of SphK2 (siSphK2-CM) failed to induce chemoresistance as potent as cells incubated with conditioned medium obtained from mock-siRNA-transfected cells (simock-CM) or SphK1-siRNA-transfected cells (siSphK1-CM; Fig. 4D).

Experiments thus far indicate that activation of SphK2 under hypoxia is a prerequisite for the release of S1P under hypoxia, thus provoking a chemoresistant phenotype in A549 cells via autocrine or paracrine mechanisms. With the following experiments, we asked which S1P receptor mediates protection in response to conditioned medium and investigated intracellular signaling pathways conveying chemoresistance. We used the S1P receptor antagonists VPC23019 (to block S1P1/S1P3) and JTE013 (to block S1P2). Only coincubation of conditioned medium with 1 μmol/L VPC23019 diminished the protective principle of CM(hypoxia), which points to S1P1 or S1P3 being involved (Fig. 5A). We then focused on signaling pathways downstream of S1P1/S1P3 receptor activation. Inhibition of major signaling cascades by LY294002 (PI3K), SB203580 (p38 MAPK), U0126 (p42/44 MAPK), or inhibitors of the JAK/STAT pathway showed that only inhibition of p42/44 MAPK by U0126 interfered with protection in response to CM(hypoxia) or Lip-CM(hypoxia). Coincubating cells with CM(hypoxia) or Lip-CM(hypoxia), which both protect cells from cell death, and 5 μmol/L U0126 sensitized cells toward etoposide-induced apoptosis (Fig. 5B). To confirm that p42/44 MAPK signaling is activated on CM(hypoxia) and Lip-CM(hypoxia), we checked phosphorylation (that is, activation) of p42/44 MAPK on treatment of A549 cells with S1P, CM(hypoxia) or Lip-CM(hypoxia). As expected, activation of p42/44 MAPK occurred after treatment of cells for 15 min with 1 μmol/L S1P. Exposing cells to CM(hypoxia) or Lip-CM(hypoxia) also produced robust p42/44 MAPK phosphorylation at 5 to 30 min, whereas the amount of total p42/44 MAPK protein and β-tubulin remained unaltered (Fig. 5C).

**Discussion**

Growing tumors require sufficient blood and nutrient supply. HIF-1 is activated to support the development and progression of tumors by releasing proangiogenic and proproliferative cytokines and/or growth factors (3, 36, 37). Another characteristic feature of cancer cells is their resistance toward chemotherapeutic agents, a process that can be promoted by intratumoral hypoxia (2, 8).

In contrast to published data showing that hypoxia failed to protect A549 cells from etoposide-induced cell death (38), we corroborated our previous results showing the appearance of a chemoresistant phenotype induced by hypoxia (5). These differences can be explained by discrepancies in treatment conditions. Cosse et al. incubated cells for 16 h under serum-free conditions in the presence of 50 μmol/L etoposide without hypoxic preincubations (5). In contrast, we preconditioned cells for 24 h under hypoxia before short-time (4 h) etoposide...
treatments. We now addressed the role of S1P as a potential mediator contributing to chemoresistance under hypoxia. Conditioned medium obtained from cells incubated under hypoxia contained a lipid that protected A549 cells from cell death via autocrine and/or paracrine mechanisms. Circumstantial evidence supports the concept that S1P is an effective mediator of chemoresistance, with the notion that SphK2 produces S1P under hypoxia. In A549 cells, both SphK isoforms are active and contribute to cell viability under unstimulated conditions. We noticed a rapid increase of SphK2 protein and activity within 1 to 4 h under hypoxia. A recent publication by Anelli et al. showed up-regulation of SphK1-mRNA and protein and the release of S1P in a HIF-2α-dependent manner (33). In endothelial cells, Schwalm et al. noticed a rapid and long-lasting activation of SphK1 under hypoxia (2-24 h), which was associated with HIF-1α- and HIF-2α-dependent signaling (32). More recently, activation of SphK1 under hypoxia was related to reactive oxygen species formation, which in turn facilitated stabilization of HIF-1α (34). In A549 cells, we excluded the HIF system to regulate SphK2, because CoCl₂, a potent inducer of HIF activity, showed no respective activity. In addition, transcriptional regulation of SphK2 under hypoxia is unlikely because we observed induction of SphK2 within 1 h of hypoxia. The fast response may point to mechanisms of mRNA stability and translation being involved. Interestingly enough, mRNA stability regulation of SphK1 has recently been reported to account for v-Src-mediated SphK1 overexpression (39), whereas information for SphK2 is lacking thus far. Moreover, one has to keep in mind that cancer cells such as A549 often harbor functional mutations in signaling pathways (e.g., of PI3K), which are responsive to hypoxia (9, 40). Hypoxia therefore may enhance mRNA transcription and translation or affect mRNA stability to increase SphK2. Alternatively, hypoxia stimulates the enzymatic activity of SphKs to increase the S1P concentration. These multiple options of hypoxic regulation may explain expression and/or activation of either SphK1 or SphK2 under various experimental settings, such as long-lasting hypoxic exposure periods (32), using CoCl₂ rather than authentic hypoxia (33) or referring to HIF-independent regulation of SphK activity as seen by Ader et al. (34) and our study.

Besides SphK2 protein expression, we observed an increase in SphK2 activity under short-time hypoxia, which correlated with an increase of extracellular S1P. It is discussed that several stimuli increased SphK activity most likely by achieving post-translational protein modification. Tumor necrosis factor-α triggered phosphorylation of SphK1 by p42/44 MAPK (41), whereas epidermal growth factor induced a protein kinase C- dependent phosphorylation and the subsequent translocation of
SphK1 to the plasma membrane (42). Phorbol 12-myristate 13-acetate triggered p42/44 MAPK and protein kinase D to phosphorylate SphK2, thus enhancing its catalytic activity and mediating its nuclear export (25, 26). Activation of signaling pathways such as PI3K, extracellular signal-regulated kinase 1/2, or p38 MAPK, which are active under hypoxia (9, 40), might therefore add to SphK2 regulation. Activation of SphK2 as seen in our study under hypoxia was cytoprotective rather than cell destructive as was observed before based on forced SphK2 overexpression (14). Differences might not only be the amount of protein being expressed by forced overexpression versus a moderated hypoxic increase but also different localization of the enzyme or the release of S1P to the extracellular space under hypoxia, which in turn can protect cells. Compensatory mechanisms effective under hypoxia may operate as well, as it is known that cells generally tolerate hypoxia very well without entering cell death by apoptosis. Along with SphK2 activation, we noticed an increase of S1P in supernatants of hypoxia-treated cells. There is little question that S1P outside cells acts cytoprotective, even being considered an essential growth-promoting factor that contributes to tumor progression (43). In this regard, the source of S1P, being either SphK1 or SphK2, should be irrelevant as long as generated S1P is exported from cells. Extracellular S1P transmits signals via G-protein-coupled receptors, of which S1P1/S1P5 have been identified. S1P receptor activation and coupling to Gi, Gq, or G12/G13 allows a crosstalk between separate G-proteins, which facilitates a fine-tuning of signals transmitted by S1P (44). Experiments using receptor antagonists point to S1P1/S1P3 being involved. Activation of PI3K and p42/44 MAPK are two pathways known to be activated by S1P (31), with the notion that these pathways also promote cell survival. Kang et al. reported up-regulation of cFLIP and phosphorylation of Bad downstream of PI3K/Akt signaling (45). We showed previously that S1P released from apoptotic cells enhanced macrophage survival via PI3K, p42/44 MAPK, and Ca2+ signaling, caused Bcl-2 and Bcl-XL expression, and inactivated proapoptotic Bad (46). This signaling cascade was, at least in part, conveyed via the S1P1 receptor. In terms of cell viability, S1P1 and S1P2 are the most important S1P receptor isoforms transmitting survival signals (31). Because only inhibition of S1P1/S1P3, but not S1P2, abrogated CM(hypoxia)-mediated protection, S1P-dependent survival is likely to be transmitted via S1P1 in A549 cells. The fact that S1P,

**FIGURE 4.** Role of SphK2 in promoting chemoresistance under hypoxia and by CM(hypoxia). A. Media were obtained from cells incubated for 24 h under hypoxia [CM(hypoxia)] or under hypoxia in the presence of 5 μmol/L DMS [CM(hypoxia/DMS)]. Naive cells were incubated for 24 h under normoxia with CM(hypoxia) or CM(hypoxia/DMS) and 1 μmol/L S1P followed by the addition of etoposide. Caspase-3 activity of cells treated with etoposide under normoxia was set to 100% and relative protection from apoptosis was determined. B. Quantification of SphK2-mRNA content of A549 cells. Cells remain untreated or were transfected with mock-siRNA, SphK1-siRNA, or SphK2-siRNA followed by incubations for 4 h under hypoxia. Basal SphK2-mRNA expression under normoxia was set to 100% and relative SphK2-mRNA expression was determined. Asterisks, significant difference compared with normoxic control. C. Cells remained untreated or were transfected with mock-siRNA, SphK1-siRNA, or SphK2-siRNA and preincubated under normoxia or hypoxia for 24 h followed by etoposide treatment. Caspase-3 activity was determined as described above. D. A549 cells remained as controls or were incubated with conditioned medium for 24 h under normoxia. Conditioned medium was generated under hypoxia by untreated cells [CM(hypoxia)], mock-siRNA-transfected cells (simock-CM), SphK1-siRNA-transfected cells (siSphK1-CM), or SphK2-siRNA-transfected cells (siSphK2-CM) followed by the addition of etoposide. Caspase-3 activity was determined as described above. Mean ± SE. *, p < 0.05.
CM(hypoxia), and lipid extract [Lip-CM(hypoxia)] induced phosphorylation (activation of p42/44 MAPK), a signaling pathway, which can be activated via Gi proteins downstream of S1P$_1$ receptors, supports this notion.

We provide evidence that hypoxia induced SphK2 expression, enhanced its activity, and promoted S1P formation. Extracellular S1P contributed to chemoresistance via S1P$_1$/S1P$_3$ receptors and p42/44 MAPK-dependent signaling pathways. We have shown previously that cyclooxygenase-2 is up-regulated under hypoxia in a HIF-1-independent fashion (5). It is interesting to note that S1P is a potent inducer of cyclooxygenase-2 expression (22, 47), thus opening the possibility that S1P is responsible for cyclooxygenase-2 induction and the formation of antiapoptotic prostaglandin E$_2$ under hypoxia. This might help to understand how signaling pathways cooperate in cell protection within hypoxic tumor regions.

Materials and Methods

Cell Culture

A549 human lung carcinoma cells were maintained in DMEM/Ham’s F-12 (1:1) supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 1% L-glutamine, and 10% FCS. THP-1 human monocytes were cultured in RPMI 1640 supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 1% L-glutamine, and 10% FCS. Differentiation of THP-1 monocytes to macrophages was done by treatment with 50 nmol/L 12-O-tetradecanoyl-phorbol-13-acetate for 16 h. Cells were incubated in a humidified atmosphere (21% O$_2$; 5% CO$_2$) and kept at a confluence of ≤80%. Hypoxic incubations were done in an InVivo2 400 Hypoxia Working Station (Biotrace) at 1% O$_2$, 94% N$_2$, and 5% CO$_2$. All cell lines where regularly tested to be free of Mycoplasma.

Reagents

All chemicals were of highest grade of purity. U0126, etoposide, staurosporine, Ac-DEVD-Amc, and DMS were purchased from Axxora. S1P (d-erythro-S1P), NBD-sphingosine, VPC23019, and JTE013 were from Avanti Polar Lipids. SphK1 and SphK2 antibodies were purchased from Abgent. Antibodies against phospho-p42/44 (Thr202/Tyr204) and p42/44 were from Cell Signaling Technology. The HIF-1α antibody was from BD Biosciences, whereas the β-tubulin and actin antibodies were from Sigma-Aldrich. Horseradish peroxidase-labeled secondary antibodies were from Amersham Biosciences and fluorescence-labeled secondary antibodies were from Rockland Immunochemicals.

Preparation of Conditioned Medium and Extraction of Phospholipids

Cells were cultured to 80% confluence in gas-permeable cell culture flasks and incubated for 24 h under normoxia (21% O$_2$) or hypoxia (1% O$_2$). If applicable, cells were stimulated as indicated. Thereafter, medium was collected, centrifuged

![FIGURE 5. S1P1/S1P3 mediate chemoresistance via extracellular signal-regulated kinase 1/2 signaling in response to conditioned medium. A, A549 cells were incubated for 24 h under normoxia or with CM(hypoxia) in the presence or absence of either 100 nmol/L JTE013 (S1P$_1$ antagonist) or 1 μmol/L VPC23019 (S1P$_1$/S1P$_3$ antagonist) before the induction of apoptosis with etoposide. Caspase-3 activity of cells treated with etoposide under normoxia was set to 100% and relative protection from apoptosis was determined. B, The specific p42/44 MAPK inhibitor U0126 (5 μmol/L) was added 30 min before incubating cells with either CM(hypoxia) or Lip-CM(hypoxia) for 24 h under normoxia followed by etoposide treatment. Caspase-3 activity was determined as described above. Mean ± SE; *, P < 0.05. C, Western analysis in A549 cells treated with S1P (1 μmol/L), CM(hypoxia), or Lip-CM(hypoxia) under normoxia for 5 to 30 min. Phospho-p42/44 (Thr202/Tyr204), total p42/44, and β-tubulin were detected by Western blot analysis. Representative of three experiments.](mcr.aacrjournals.org)
(5 min, 4°C, 500 × g) and filtered (0.22 μm) to remove cell debris, transferred into fresh tubes, and stored at 4°C. For phospholipid extraction, 1 mL conditioned medium was mixed with 3.75 mL chloroform/methanol/12 N HCl (v/v/v, 2/4/0.1) and vortexed vigorously. Thereafter, 1.25 mL chloroform was added followed by vortexing. Finally, 1 mL double-distilled H2O was added followed by subsequent agitation for 5 min and centrifugation for 10 min at 1,000 rpm to achieve phase separation. The lower chloroform phase was transferred into a fresh tube and traces of water were removed by adding sodium sulfate. The lipid phase was filtered and concentrated using a speed-vac and extracts were resuspended in ethanol. The volume of lipid extract used for stimulation of the cells contained the amount of lipids extracted from 2 mL conditioned medium.

Transfection with siRNA

Cells [3 × 10^9 (caspase-3 activity assay) or 1 × 10^9 (mRNA extraction)] were plated in cell culture dishes. The following day, 100 nmol/L of either specific siRNA or siCONTROL nontargeting Duplex 1 (mock-siRNA) was transfected with DharmaFECT1 transfection reagent following the manufacturer's guideline. Transfected cells were incubated under normoxia for 48 h in medium containing the transfection mixture. Before the experiments, medium was changed and cells were stimulated as indicated. DharmaFECT1 and siCONTROL nontargeting Duplex 1 were purchased from Dharmacon. Validated SphK1- or SphK2-specific siRNA was purchased from Qiagen.

Western Blot Analysis

Briefly, cells (1 × 10^6 per 10 cm dish) were incubated as indicated, scraped off the plates, lysed in 100 μL lysis buffer (100 nmol/L HEPES, 0.1% CHAPS, 10% sucrose, protease inhibitor mix, 1 mL/100 μL DTT), sonicated on ice (10 s), and centrifuged (15,000 × g, 15 min). Protein (120 μg) was mixed with 4× SDS-PAGE sample buffer [125 μmol/L Tris-HCl, 2% SDS, 20% glycerine, 1 mL/100 μL DTT, 0.002% bromophenol blue (pH 6.9)], denatured for 5 min, and centrifuged (15,000 × g, 5 min). Protein was separated by 10% SDS-PAGE. Gels were washed with blotting buffer [25 mmol/L Tris-HCl, 192 mM glycerine, 20% methanol (pH 8.3)] for 5 min. Gels were blotted on Hybond C extra nitrocellulose membrane (Amersham Biosciences) by a semidy electromotrafic transfer cell (Bio-Rad). Unspecific binding was blocked with 5% milk/TBS [50 mmol/L Tris-HCl, 140 mmol/L NaCl, 0.05% Tween 20 (pH 7.2)] for 1 h. All antibodies used were incubated following the manufacturer’s guidelines. Afterwards, membranes were washed three times for 5 min each with PBS. For protein detection, blots were incubated with horseradish peroxidase-labeled secondary antibodies (1:2,000 in 5% milk/Tween 20-TBS) for 2 h and washed once for 5 min with Tween 20-TBS and twice for 5 min each with PBS followed by enhanced chemiluminescence or fluorescence detection using Odyssey infrared imaging system (LI-COR Biotechnology).

Caspase-3 Activity Assay

Apoptosis was induced under serum-free conditions using either 150 μmol/L etoposide or 1 μg/mL staurosporine for 4 h (A549) or 1 μg/mL staurosporine for 8 h (THP-1). Caspase-3 activity was quantified following the cleavage of Ac-DEVD-AMC as described previously (5). Briefly, cells were resuspended in assay buffer (100 nmol/L HEPES, 0.1% CHAPS, 10% sucrose, 1 mL/100 μL DTT), sonicated on ice (15 s), and centrifuged at 15,000 × g for 15 min. The cell supernatant (50 μg) was diluted with assay buffer to a final volume of 140 μL, Ac-DEVD-AMc substrate (0.2 mmol/L) was added, and caspase-3 activity was determined for 1 h at 30°C using the Mithras LB940 multimode reader (Berthold; extinction, 360 nm; emission, 460 nm).

SphK Activity Assay

Activity of SphKs was followed as phosphorylation of NBD-sphingosine to NBD-S1P as described previously (48). For preparation of whole-cell lysates, cells were harvested, washed with ice-cold PBS, and resolved in 100 μL ice-cold SphK lysis buffer [20 mmol/L Tris-HCl, 20% (v/v) glycerol, 1 mmol/L l-mercaptoethanol, 1 mL/100 μL EDTA, 15 mmol/L NaF, 40 mmol/L β-glycerophosphate, protease inhibitor mix (pH 7.4)]. Subsequently, lysates were sonicated on ice for 5 s followed by centrifugation (12,000 × g, 30 min, 4°C). The supernatants (10 μL) were then added to 100 μL SphK assay buffer [SphK1: 50 mmol/L HEPES, 15 mmol/L MgCl2, 0.5% Triton X-100, 10 mmol/L NaF, 1.5 mmol/L semicarbazide (pH 7.4); SphK2: 50 mmol/L HEPES, 15 mmol/L MgCl2, 1 mmol/L KCl, 10 mmol/L NaF, 1.5 mmol/L semicarbazide (pH 7.4)], together with 10 μmol/L NBD-sphingosine and 1 mmol/L ATP, for 2 h at 30°C. Addition of 100 μL alkaline potassium phosphate buffer followed by chloroform/methanol/HCl (500 μL, 2:1:0.1, v/v/v) allowed to isolate the product NBD-S1P in the aqueous phase. The aqueous phase (100 μL) was then analyzed for NBD-S1P-dependent fluorescence in the Mithras LB 940 multimode (extinction, 485 nm; emission, 538 nm).

Quantification of S1P

Quantification of S1P in cell culture supernatants was achieved by the S1P ELISA kit (Echelon Biosciences). Supernatants were collected from A549 cells and incubated under normoxia (4 h) or hypoxia (2–4 h) under serum-free conditions. Before incubation, cells were washed twice in PBS to remove traces of serum. Additionally, we determined S1P concentrations in CM(hypoxia) or CM(hypoxia/DMS). Quantification of S1P was done following the manufacturer’s guidelines. Absorbance (450 nm) was measured by using the Apollo-8 plate reader (Berthold).

Statistical Analysis

Each experiment was done at least three times. Representative blots are shown. Data are represented as mean ± SE. Statistical analysis was done using a one-way ANOVA combined with Tukey’s post-test (GraphPad Prism version 4.00). Unless indicated otherwise, differences were considered significant at P ≤ 0.05.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Hypoxia-Induced S1P Mediates Chemoresistance

References

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