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The roles of sphingosine kinases 1 and 2 in regulating the Warburg effect in prostate cancer cells

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ABSTRACT

Two isoforms of sphingosine kinase, SK1 and SK2, catalyze the formation of the bioactive lipid sphingosine 1-phosphate (S1P) in mammalian cells. We have previously shown that treatment of androgen-sensitive LNCaP prostate cancer cells with a non-selective SK isoform inhibitor, 2-(*p*-hydroxyanilino)-4-(*p*-chlorophenyl)thiazole (SKi), induces the proteasomal degradation of SK1. This is concomitant with a significant increase in C22:0-ceramide and sphingosine levels and a reduction in S1P levels, resulting in the apoptosis of LNCaP cells. In contrast, we show here that a SK2-selective inhibitor, (*R*)-FTY720 methyl ether (ROME), increases sphingosine and decreases S1P levels but has no effect on ceramide levels and does not induce apoptosis in LNCaP cells. We also show that several glycolytic metabolites and (*R*)-*S*-lactoylglutathione are increased upon treatment of LNCaP cells with SKi, which induces the proteasomal degradation of c-Myc. These changes reflect an indirect antagonism of the Warburg effect. LNCaP cells also respond to SKi by diverting glucose 6-phosphate into the pentose phosphate pathway to provide NADPH, which serves as an antioxidant to counter an oxidative stress response. SKi also promotes the formation of a novel pro-apoptotic molecule called diadenosine 5′,5‴-P^1,P^3-triphosphate (Ap3A), which binds to the tumor suppressor fragile histidine triad protein (FHIT). In contrast, the SK2-selective inhibitor, ROME, induces a reduction in some glycolytic metabolites and does not affect oxidative stress. We conclude that SK1 functions to increase the stability of c-Myc and suppresses Ap3A formation, which might maintain the Warburg effect and cell survival, while SK2 exhibits a non-overlapping function.

1. Introduction

Sphingosine kinase is a lipid kinase that has two isoforms, SK1 and SK2, which are encoded by distinct genes and differ in their biochemical properties, subcellular localization, and function [1]. SK1 mRNA transcript and/or protein expression are increased in various human tumors [1]. These findings support the hypothesis that high SK1 expression in cancer cells confers positive selection to these cells. Indeed, siRNA knockdown of SK1 reduces proliferation of glioblastoma cells [2] and androgen-independent PC-3 prostate cancer cells [3]. In addition, large vascularized resistant tumors are formed when cancer cells over-expressing SK1 are injected or implanted into mice [1,4]. SK1 is also a sensor that confers chemotherapeutic resistance, promoting the survival of cancer cells in the presence of certain anti-cancer agents [5].

We have previously reported that the treatment of cancer cells for 24–48 h with a non-selective SK isoform inhibitor, SKi (2-(*p*-hydroxyanilino)-4-(*p*-chlorophenyl)thiazole), activates the proteasome to remove SK1 from breast and prostate cancer cells [5,6]. Ubiquitin-proteasomal degradation of SK1 occurs by a ceramide-dependent mechanism after an initial inhibition of SK activity [5]. This leads to a reduction in the intracellular S1P level and an elevation of C22:0-ceramide level, which is thought to induce apoptosis [5]. Importantly, the apoptotic effect is 'on-target' as it can be recapitulated by siRNA knockdown of SK1 in breast cancer cells [7]. The apoptotic effect induced after proteasomal degradation of SK1 is consistent with the loss of S1P, which is known to regulate apoptotic proteins, such as Bax and Bad [8].

Abbreviations: Ap3A, diadenosine triphosphate; ERK, extracellular signal regulated kinase; FHIT, fragile histidine triad protein; GSSG, oxidized glutathione; GSH, reduced glutathione; LC3, microtubule associated protein 1 light chain 3; NAC, N-acetyl-cysteine; NADPH, nicotinamide dinucleotide phosphate; ND, not detected; PARP, polyADPribose polymerase; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SK1, sphingosine kinase 1; S1P, sphingosine 1-phosphate.

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Prostate cancer cells express two N-terminal variants of SK1 [5], termed SK1a and SK1b. SK1b has an 86 N-terminal amino-acid extension compared with SK1a, and is also sometimes referred to as SK1c. The properties of the SK1b variant have not been well characterized yet its presence in cancer cells is important as it confers a cell survival advantage [5]. SK1b is very stable, being resistant to the protein synthesis inhibitor cycloheximide and siRNA knockdown in prostate cancer cells [5]. However, SK1b is sensitive to SK inhibitor-induced proteasomal degradation in androgen-sensitive prostate cancer cells [5,6]. Therefore, SK inhibitors represent an effective method for reducing the amount of this protein in these cells to facilitate meaningful study of its role in cancer biology.

There is emerging evidence for an important role of SK2 in cancer. This is exemplified by the finding that the siRNA knockdown of SK2 enhances doxorubicin-induced apoptosis of breast or colon cancer cells [9] and reduces cancer cell proliferation and migration/invasion [10]. However, there is also evidence that SK1 and SK2 have opposing actions [11]. Therefore, there is a need to develop isoform-selective inhibitors in order to better define the role of these enzymes in cancer cells. (*R*)-FTY720-OMe (ROME) is a specific, enantioselective, competitive (with sphingosine) inhibitor of SK2 [12]. Treatment of MCF-7 cells with ROME prevents actin enrichment into lamellipodia in response to S1P, suggesting that metastasis can be inhibited [12]. The K_i values for inhibition of SK2 by ROME and a second SK2-specific inhibitor, ABC294640, are very similar (16.5 μM and 10.0 μM, respectively [12,13]).

A major phenotypic hallmark of cancer cells is their obligate need to convert glucose to lactate under aerobic conditions (the Warburg effect) to produce ATP required for cancer cell growth [14]. We hypothesized that indirect inhibition of the Warburg effect by targeting the SK isoforms might provide an approach for selectively attacking cancer cells that display 'addiction' to SK. Therefore, we decided to investigate the roles of SK1 and SK2 in regulating the metabolome of androgen-sensitive LNCaP prostate cancer cells in which we have previously extensively characterized the effect of SK inhibitors [5,6,11]. These studies have enabled determination of the roles of SK1 and SK2 in regulating the Warburg effect, thereby maintaining survival and immortality of cancer cells.

2. Experimental procedures

2.1. Materials

All general biochemicals were from Sigma (Poole, UK). RPMI 1640, OptiMEM, penicillin-streptomycin (10,000 U/ml penicillin and 10,000 μg/ml streptomycin), L-glutamine and Lipofectamine 2000™ were from Invitrogen (Paisley, UK). European fetal calf serum (EFCS) was purchased from Sera Laboratories (Haywards Heath, UK). SKi was from Merck Biosciences (Nottingham, UK). MG132 was from Enzo Life Sciences (Exeter, UK). ROME was synthesized as described previously [12]. Antibodies were obtained as follows: anti-actin from Sigma (Poole, UK); anti-myc from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-ERK2 from BD Transduction Laboratories (Oxford, UK). Anti-SK1 antibody was a kind gift from Dr Andrea Huwiler (University of Berne, Switzerland). LNCaP cells were gifts from Professor Hing Leung (Beatson Institute, Glasgow). Myc-tagged SK2 plasmid construct was generated as described previously [16].

2.2. Cell culture

Human androgen-sensitive LNCaP cells were maintained in RPMI 1640 medium supplemented with 10% EFCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% L-glutamine. All cells were maintained in a humidified atmosphere at 37 °C with 5% CO_2. LNCaP cells were transiently transfected with a myc-tagged SK2-pcDNA4 plasmid construct using Lipofectamine2000™ reagent according to the manufacturer's instructions.

2.3. Preparation of whole cell extracts

Cell extracts for SDS-PAGE and Western blot analysis were prepared by washing treated cells with 5 ml of PBS and then re-suspending the cell pellets in whole cell lysis buffer [(137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% v/v NP40, 10% v/v glycerol, 20 mM Tris) (pH 8.0) containing 0.2 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.5 mM Na_3VO_4, 100 μM NaF, and 10 mM β-glycerophosphate]. Samples were repeatedly (×6) passed through a 23-gauge needle using a syringe and rotated for 30 min at 4 °C to allow for efficient lysis. Cell debris was pelleted by centrifugation at 22,000 ×*g* for 10 min at 4 °C, and the supernatant (whole cell extract) was collected. The protein content was measured using the Pierce BCA assay kit (Fisher Scientific, Loughborough, UK). For each sample, 10–20 μg of protein was added to sample buffer [125 mM Tris, pH 6.7, 0.5 mM $Na_4P_2O_7$, 1.25 mM EDTA, 0.5% w/v SDS containing 1.25% v/v glycerol, 0.06% w/v bromophenol blue, and 50 mM dithiothreitol], and used for SDS-PAGE and western blotting using anti-myc, anti-cyclin D1, anti-actin, anti-ERK2, and anti-PARP antibodies. For the detection of LC3 conversion (LC3-I to LC3-II) by immunoblotting for autophagy analysis, cells were washed with ice-cold PBS and resuspended in TNTE lysis buffer [20 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, 0.3% Triton X-100, 50 μg/ml PMSF, Protease Inhibitor Cocktail; pH 7.5]. Samples were repeatedly (×10) passed through a 0.24-mm gauge needle and syringe and left for 5 min at 4 °C to allow for efficient lysis. After cell debris was pelleted by centrifugation at 22,000×*g* for 10 min at 4 °C, the supernatant was collected and the protein content was measured. For each sample, 80 μg of protein was combined with sample buffer and subjected to SDS-PAGE and western blotting using anti-LC3 antibodies.

2.4. Proteasome activity assays

Proteasome activity was measured in cells using a Proteasome Glo Chymotrypsin-Like Cell-based assay kit (Promega) as per the manufacturer's instructions.

2.5. Analysis of sphingoid base 1-phosphates and ceramides

Analyses of sphingoid base 1-phosphates, ceramides, and sphingoid bases were performed by electrospray ionization tandem mass spectrometry (ESI-LC/MS/MS) on an AB Sciex 5500 QTRAP hybrid triple quadrupole linear ion-trap mass spectrometers (Applied Biosystems, Foster City, CA) equipped with a TurboIonSpray ionization source interfaced with an automated Agilent 1200 series liquid chromatograph (Agilent Technologies, Wilmington, DE). S1P and dihydro-S1P (DHS1P) were analyzed as bis-*O*-acetylated derivatives with C17-S1P as the internal standard employing reverse-phase HPLC separation, negative ion ESI, and Multiple Reaction Monitoring (MRM) analysis as described in [15]. Ceramides were analyzed with C17-ceramide as an internal standard using reverse-phase HPLC separation, positive ion ESI, and MRM analysis as described in [16]. Briefly, resolution of sphingoid bases was achieved on a Discovery C18 column (2.1×50 mm, 5 μm particle size, Supelco, Bellefonte, PA) and a gradient from methanol/water/formic acid (61:38:1, v/v/v) with 5 mM ammonium formate to methanol/acetonitrile/formic acid (39:60:1, v/v/v) with 5 mM ammonium formate at a flow rate of 0.5 ml/min. The MRM transitions employed for detection of sphingoid bases were as follows: *m/z* 286>268 (C17-Sph, internal standard); *m/z* 300>282 (Sph); and *m/z* 302>284 (DHSph).