The sphingosine kinase-1 survival pathway is a molecular target for the tumor-suppressive tea and wine polyphenols in prostate cancer

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ABSTRACT The sphingosine kinase-1/sphingosine 1-phosphate (SphK1/S1P) pathway has been associated with cancer promotion and progression and resistance to treatments in a number of cancers, including prostate adenocarcinoma. Here we provide the first evidence that dietary agents, namely, epigallocatechin gallate (EGCg, IC50 ~75 μM), resveratrol (IC50 ~40 μM), or a mixture of polyphenols from green tea [polyphenon E (PPE), IC50 ~70 μM] or grapevine extract (vineteatrol, IC50 ~30 μM), impede prostate cancer cell growth in vitro and in vivo by inhibiting the SphK1/S1P pathway. We establish that SphK1 is a downstream effector of the ERK/phospholipase D (PLD) pathway, which is inhibited by green tea and wine polyphenols. Enforced expression of SphK1 impaired the ability of green tea and wine polyphenols, as well as pharmacological inhibitors of PLD and ERK activities, to induce apoptosis in PC-3 and C4-2B cells. The therapeutic efficacy of these polyphenols on tumor growth and the SphK1/S1P pathway were confirmed in animals using a heterotopic PC-3 tumor in place model. PC-3/SphK1 cells implanted in animals developed larger tumors and resistance to treatment with polyphenols. Furthermore, using an orthotopic PC-3/GFP model, the chemopreventive effect of an EGCg or PPE diet was associated with SphK1 inhibition, a decrease in primary tumor volume, and occurrence and number of metastases. These results provide the first demonstration that the prosurvival, antiproapoptotic SphK1/S1P pathway represents a target of dietary green tea and wine polyphenols in cancer.—Brizuela, L., Dayon, A., Doumerc, N., Ader, I., Golzio, M., Izard, J.-C., Hara, Y., Malavaud, B., Cuvillier, O. The sphingosine kinase-1 survival pathway is a molecular target for the tumor-suppressive tea and wine polyphenols in prostate cancer. FASEB J. 24, 3882–3894 (2010). www.fasebj.org

Key Words: resveratrol • EGCg • phospholipase D • ERK1/2

The high prevalence, long latency, and significant mortality associated with prostate cancer make it a suitable target for primary prevention (1). Epidemiological, preclinical, and early clinical investigations suggest that polyphenolic phytochemicals have the potential to modify many of the acquired characteristics that are required for initiation of carcinogenesis, tumor promotion, and progression in many types of cancers, including prostate cancer (2). Of this class of compounds, epigallocatechin gallate (EGCg) from tea and resveratrol from red grapes have gained recognition as important chemopreventive agents and as modulators of tumor cell response to chemotherapy (3, 4). In vitro and in vivo studies have demonstrated that EGCg and resveratrol affect a wide array of molecular pathways, resulting in inhibition of cell growth, invasion, angiogenesis, and metastasis (5). In prostate cancer, EGCg and resveratrol alter numerous intracellular pathways, including inhibition of ERK1/2- and Akt-mediated signaling, inhibition of PMA-dependent PKC activation, alteration of Bcl-2 family members ratio, and activation of caspases (6). Xenograft tumor models as well as transgenic animal studies have shown that green tea or wine polyphenols can decrease the tumorigenic potential of prostate cancer (7–10).

Sphingosine 1-phosphate (SIP) is a bioactive lipid involved in tumor cell growth, survival, invasion, and neoangiogenesis (11). SIP content in cells is low and is kept under control through a delicately regulated balance between its synthesis and its degradation (11). The balance between the levels of SIP and its metabolic precursors ceramide and sphingosine has been suggested to be a switch determining whether a cell proliferates or dies (12). The master regulator of this ceramide/SIP balance is the sphingosine kinase-1 (SphK1), an enzyme converting the proapoptotic sphingosine into the prosurvival S1P. SphK1 activity can be stimulated by many agonists and inhibited by green tea and wine polyphenols. Enforced expression of SphK1 impaired the ability of green tea and wine polyphenols, as well as pharmacological inhibitors of PLD and ERK activities, to induce apoptosis in PC-3 and C4-2B cells. The therapeutic efficacy of these polyphenols on tumor growth and the SphK1/SIP pathway were confirmed in animals using a heterotopic PC-3 tumor in place model. PC-3/SphK1 cells implanted in animals developed larger tumors and resistance to treatment with polyphenols. Furthermore, using an orthotopic PC-3/GFP model, the chemopreventive effect of an EGCg or PPE diet was associated with SphK1 inhibition, a decrease in primary tumor volume, and occurrence and number of metastases. These results provide the first demonstration that the prosurvival, antiproapoptotic SphK1/SIP pathway represents a target of dietary green tea and wine polyphenols in cancer.—Brizuela, L., Dayon, A., Doumerc, N., Ader, I., Golzio, M., Izard, J.-C., Hara, Y., Malavaud, B., Cuvillier, O. The sphingosine kinase-1 survival pathway is a molecular target for the tumor-suppressive tea and wine polyphenols in prostate cancer. FASEB J. 24, 3882–3894 (2010). www.fasebj.org

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either extracellularly, by binding to 1 of the 5 cell surface G-coupled S1P receptors to drive paracrine or autocrine signaling cascades, or intracellularly by a mechanism yet to be investigated (13). Importantly, the agonist-induced S1P production, as well as its downstream effects, can be impeded by inhibiting SphK1 activity, demonstrating that SphK1 plays a crucial role in the observed effects ascribed to S1P. Multiple studies support the convincing role of SphK1 in the promotion of oncogenesis, in addition to being a cellular target for many anticancer treatments. On the one hand, since the demonstration of its oncogenic nature, SphK1 expression has been found to be upregulated in a number of solid tumors, and high SphK1 expression in glioblastoma and gastric and breast cancers has been correlated with poor survival of patients (14–16). On the other hand, anticancer regimens have been shown to down-regulate SphK1 activity in various cancer cell and animal models, suggesting that SphK1 may act as a “sensor” to anticancer therapies, whereas its enforced expression protects cancer cells from apoptosis (17).

In prostate cancer cell and animal models, the SphK1/S1P pathway is associated with resistance to chemotherapy (18, 19) and radiotherapy (20) as well as progression toward a hormone-refractory state (21). In addition, both SphK1 enzymatic activity and expression are significantly increased in patient tumor samples (as compared with normal counterparts) and correlate with markers such as prostate-specific antigen, neuroendocrine biomarkers, and tumor grade as well as with the clinical outcome after prostatectomy (unpublished results). The present study was thus conducted to evaluate the role of SphK1/S1P signaling in response to EGCg, resveratrol, or a mixture of polyphenols from green tea [polyphenon E (PPE)] or grapevine extract (vineatrol) in prostate cancer cells and animal models. We report for the first time that green tea and wine polyphenols inhibit SphK1 activity, via a novel ERK/phospholipase D (PLD)-dependent mechanism in C4-2B hormone-responsive and PC-3 hormone-refractory prostate cancer cells. In vivo experiments, using heterotopic and orthotopic xenografted PC-3 models, confirmed the contribution of SphK1 in both chemopreventive and therapeutic potential of polyphenols in prostate cancer.

MATERIALS AND METHODS

Chemicals and reagents

Culture medium, serum, and antibiotics were from Invitrogen. [γ-32P]ATP, [9,10-3H(N)]-palmitic acid, and [methyl-3H]thymidine were from Perkin-Elmer (Courtaboeuf, France). Silica gel 60 TLC plates were from VWR (West Chester, PA, USA). Sphingosine was from Biomol (Plymouth Meeting, PA, USA). MTT and phorbol 12-myristate 13-acetate (PMA) were from Sigma-Aldrich (St. Louis, MO, USA). The MEK1/2 inhibitors, U0126 and PD98059, and sphingosine kinase inhibitor (SKI) were from Calbiochem (San Diego, CA, USA). 1,2-dipalmitoyl-sn-glycero-3-phosphethanol was from Avanti Polar Lipids (Alabaster, AL, USA).

Cell models

The PC-3 and C4-2B cell lines were from DSMZ (Braunschweig, Germany) and Viromed (Minnetonka, MN, USA), respectively. C4-2B is a castration-resistant prostate cancer cell line isolated from the bone metastasis of a mouse xenograft inoculated with C4-2 cells, a subline of LNCaP cells. Cells were cultured in RPMI 1640 containing 10% FBS at 37°C in 5% CO2 humidified incubators. FLAG epitope-tagged wild-type human SphK1 cDNA (22) was used for stable transfection using Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA). Mass pools of stable transfectant were selected with 0.4 and 0.8 mg/ml G418 for PC-3 and C4-2B cells, respectively. Empty vector- and wild-type human SphK1-transfected cells were designated as PC-5.neo, C4-2B.neo, PC-3/SphK1, and C4-2B/SphK1, respectively. A GFP-overexpressing PC-3 cell model has been previously described (18). Cell lines were routinely verified by the following tests: morphology check by microscope, growth curve analysis, and mycoplasma detection (MycoAlert; Lonza, Basel, Switzerland). All experiments were started with low-passaged cells (<20 times).

EGCg and PPE

A purified preparation of EGCg (>98% pure) and PPE, a green tea extract containing 80–98% total catechins by weight (the main component is EGCg, representing ~65% of the material), were from Mitsui-Norin (Shizuoka, Japan).

Wine polyphenols

Resveratrol and vineatrol, a grapevine extract that mostly combines resveratrol and its dimer ε-viniferin (47.5% dry weight as determined by HPLC), were prepared by Actichem (Montauban, France) from vine shoot extracts (patent no. FR2795964).

Cell viability assay

Cell growth inhibition was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) bromide assay (23).

3H-thymidine incorporation assay

PC-3 and C4-2B cells were plated in 6-well plates (2 × 10^5 and 5 × 10^5 cells/well, respectively), then cultured for 2 d in RPMI containing 10% (v/v) FBS. Cells were next treated or not with different drugs for 24 and 48 h. 3H-thymidine was added to the culture medium (1 μCi/ml) 6 h before the end of the experiment. Cells were washed once with ice-cold PBS and 3 times with 10% trichloroacetic acid to cause the precipitation of DNA and proteins. The precipitate was solubilized in 0.3 N NaOH/1% SDS, and radioactivity was measured (21).

Western blot analysis and antibodies

Rabbit anticaspase 3, mouse antiphospho-ERK1/2, and rabbit anti-ERK1/2 from CST (Danvers, MA, USA), and rabbit anti-SphK1 (22) were used as primary antibodies. Proteins were visualized by an enhanced chemiluminescence detection system (Pierce, Brébières, France) using anti-rabbit or anti-mouse.
SphK1 assay, mass measurement of ceramide, and S1P

The protocols for measurement of SphK1 activity, ceramide, and S1P amounts have been described in detail previously (24).

PLD assay

PLD activity was determined by measuring the production of \([3H]\)-phosphatidylethanol, which is the product of its transphosphatidylation activity (25). Briefly, the cells were incubated for 16 h with 0.5 \(\mu\)Ci \([9,10-^3H(N)]\)-palmitate/ml to label phosphatidylethanolamine. The radioactivity was then removed, and cells were washed 3 times with nonradioactive RPMI containing 0.2% BSA. Ethanol, at a final concentration of 1%, was added 5 min before the addition of agonists, and the cells were incubated further for 30 min, the optimal time to recover the maximal formation of phosphatidylethanolamine. Lipids were next extracted as described by Bligh and Dyer (26), except that 2 M KCl in 0.2 M HCl was added to the extraction mixture instead of water for the separation of the aqueous and organic phases. Chloroform phases were then vacuum-dried in an automatic SpeedVac concentrator (Savant USV400A; Thermo Savant, Holbrook, NY, USA) and resuspended in 50 \(\mu\l\) of chloroform. Lipids were separated by thin layer chromatography (TLC) using Silica Gel 60 plates. The TLC plates were developed with chloroform/methanol/acetic acid (9:1:1, v/v/v), and the positions of lipids were identified after staining with iodine vapor by comparison with authentic standards. The silica gel-containing radioactive lipids were quantitated by liquid scintillation counting after scraping the spots off the plates.

RNA interference experiments

Transient interference was achieved by double-stranded human SphK1–specific small interfering RNA or alleatory sequence scrambled siRNA (Eurogentec, Angers, France) as previously reported (27). SiRNA transfection was carried out with the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions.

Animals

Male NMRI/Nu (nu/nu) 6-wk-old mice were obtained from Elevage Janvier (Le Genest Saint Isle, France). Mice were housed in a barrier facility of high-efficiency particulate air-filtered racks. At 7–8 wk of age, the animals were used in accordance with the principles and procedures outlined in Council Directive 86/809/EEC. The Institut Fédératif de Recherche Bio-médicale de Toulouse Animal Care and Use Committee approved all animal studies.

Heterotopic implantation of PC-3 prostate cancer cells

A suspension of \(1 \times 10^6\) PC-3 cells in a 40-\(\mu\l\) volume was injected s.c. into the right posterior hind leg with a syringe equipped with 29-gauge needle. After cell inoculation, the animals were kept under pathogen-free conditions, with standard diet and water available \(ad libitum\) during tumor development. The growth of the primary tumor was quantified by \(in situ\) caliper measurements, and tumor volume \((mm^3)\) was calculated using the following formula: \(L \times W^2 \times \pi / 6\), where \(L\) is the length (\(mm\)) and \(W\) is the width (\(mm\)). Tumors were allowed to grow for 10 d (until a volume of 100–130 mm\(^3\)) before treatment.

Orthotopic implantation of PC-3/GFP prostate cancer cells, autopsy, and \(in vivo\) fluorescence imaging

Intraprostatic human prostate cancer xenografts were established in nude mice by surgical orthotopic implantation. Mice were anesthetized by isoflurane inhalation and placed in the supine position. A lower midline abdominal incision was made, and a 20-\(\mu\l\) tumor cell suspension \((1 \times 10^6\) cells\) was injected into the dorsal lobe of the prostate using a 30-gauge needle and glass syringe. After implantation, the surgical wound was closed in 2 layers with 4-0 Dexon interrupted sutures. All procedures were performed with a dissecting microscope. Autopsy and \(in vivo\) fluorescence imaging were conducted as previously detailed (18).

Statistical analysis

The statistical significance of differences between the means of 2 groups was evaluated using the unpaired Student’s \(t\) or the 1-way analysis of variance (ANOVA) tests. The frequencies of metastasis between the 2 groups were compared using Fisher’s exact test. Differences in the number of metastases per group were examined using a nonparametric Wilcoxon rank sum test. All statistical tests were 2-sided, and the level of significance was set at \(P < 0.05\). Calculations were done using Instat 3 for Macintosh (GraphPad Software, San Diego, CA, USA).

RESULTS

Green tea and wine polyphenols induce SphK1 down-regulation

As previously reported in various prostate cancer cell models, treatment with EGCg (28) or resveratrol (29) inhibited cell growth of PC-3 (Fig. 1A) and C4-2B (not shown) cells. A similar effect was observed in PC-3 (Fig. 1A) and C4-2B (not shown) cells treated with extracts of wine polyphenols (vineteatrol) and green tea polyphenols (PPE). Thymidine proliferation as-
say confirmed these results (not shown). EGCg and PPE inhibited PC-3 cell growth with an IC₅₀ value at 48 h of ~75 µM and 50 µg/ml (equivalent to ~70 µM EGCg), respectively. For resveratrol and vineatrol, IC₅₀ values at 48 h were ~10 µg/ml (40 µM) and 20 µg/ml (equivalent to ~30 µM resveratrol), respectively. Because the inhibition of the prosurvival SphK1 signaling pathway has been reported during anticancer treatments in various cancer cell systems including prostate (18–20), we next evaluated the ability of green tea and wine polyphenols (at a concentration corresponding to the IC₅₀ value at 48 h of treatment) to inhibit SphK1 activity and expression. Figure 1B demonstrate that these compounds induce a profound inhibitory effect toward SphK1 in PC-3 cells, which was significant as early as 30 min of exposure, with ~50% SphK1 inhibition obtained within 90 min to 3 h of treatment. This pattern was amplified over time, and a 72-h treatment decreased SphK1 activity by ≥75% in PC-3 (Fig. 1B) and C4-2B (not shown) cells. By altering the sphingolipid balance (12), the treatment with tea and wine polyphenols was correlated with an increase in pro-apoptotic ceramide content in both PC-3 and C4-2B cell lineages that paralleled SphK1 inhibition (not shown). Of note, the inhibition of the enzymatic activity of SphK1 preceded the diminution of its protein expression, which was seen only at later times of incubation (Fig. 1C).

SphK1 overexpression protects prostate cancer cells from the effects of green tea and wine polyphenols

Because an inhibition of SphK1 activity was observed during polyphenol-induced cell death, we next asked whether transfection of PC-3 and C4-2B cells with SphK1 might render these cells resistant to these compounds. SphK1 activity of both SphK1-overexpressing PC-3 and C4-2B cells was increased up to 1100–1300 pmol/mg protein/min, as previously reported (21). In response to green tea and wine polyphenols, the inhibition of SphK1 activity was markedly reduced in PC-3/SphK1 and C4-2B/SphK1 cells than in neo counterparts (not shown). SphK1-overexpressing PC-3 and C4-2B cells were remarkably resistant to cell death induced by resveratrol, vinea-
trol, EGCg, and PPE (Fig. 2A). Similarly, the diminution of SphK1 protein expression, observed in nontransfected cells at later times of incubation (Fig. 1C), was not seen in PC-3/SphK1 and C4-2B/SphK1 cells (Fig. 2B). The cytoprotective effect of SphK1 overexpression was associated with a strong inhibition of the processing of executioner caspase-3 (Fig. 2C), an endogenous apoptosis marker reported to be activated by green tea and wine polyphenols (30, 31).

**Green tea and wine polyphenols down-regulate SphK1 activity via the inhibition of the ERK/PLD pathway**

ERK1/2 and PLD/phosphatidic acid (PLD/PA) pathways have been previously identified as critical mediators for SphK1 activation (22, 32). We thus examined the effects of green tea and wine polyphenols on ERK1/2 and PLD/PA activities. Figure 3A shows that resveratrol, vineatrol, EGCg, and PPE induced ERK1/2 dephosphorylation in PC-3 and C4-2B cells as early as 15 min of treatment. Direct interruption of ERK1/2 signaling, by PD98059 and UO126, was seen within minutes of incubation (Fig. 4C). PD98059- and UO126-treated cells underwent a rapid decrease in SphK1 activity (Fig. 3B). Confirming that SphK1 down-regulation was a consequence of ERK1/2 inhibition, enforced expression of SphK1 rendered PC-3 and C4-2B cells significantly more resistant to PD98059 and UO-126 (Fig. 3C).

PLD has been widely reported to be activated by phorbol esters such as PMA, and, in many situations, activated PLD is regarded as a hallmark of conventional PKC activation (33). In the presence of etha-
PLD catalyzes a transphosphatidylation reaction in which H$_2$O is replaced by a primary alcohol to yield PtdEtOH. This reaction is unique to PLD and is used as a specific assay for the enzyme (34). As anticipated, PMA greatly enhanced the accumulation of PtdEtOH in PC-3 and C4-2B cells, indicative of PLD activation (Fig. 4A). Resveratrol, vineatrol, EGCg, and PPE significantly inhibited PMA-stimulated PLD activity in both prostate cancer cell lineages (Fig. 4A). The activation of PLD relied on

**Figure 3.** Inhibition of SphK1 activity by green tea and wine polyphenols is ERK1/2 dependent. A) PC-3 (left panel) and C4-2B (right panel) cells were incubated with 10 μg/ml resveratrol, 20 μg/ml vineatrol, 75 μM EGCg, or 50 μg/ml PPE for 15 min. Cells were lysed and phosphorylation of ERK 1/2 was analyzed by immunoblotting. Similar results were obtained in ≥3 independent experiments. B) PC-3 (left panel) and C4-2B (right panel) cells were treated with the pharmacological inhibitors of MEK 1/2, UO126 (10 μM), or PD98059 (20 μM) for the indicated times and tested for SphK1 activity. Columns indicate means of 5 independent experiments; bars indicate SEM. C) PC-3/neo and PC-3/SphK1 (left panel) and C4-2B/neo and C4-2B/SphK1 (right panel) cells were treated with UO126 (10 μM) or PD98059 (20 μM) for the indicated times, and cell proliferation was assessed with $^3$H-thymidine incorporation assay. Columns indicate means of 6 independent experiments; bars indicate SEM. *P < 0.05, **P < 0.01, ***P < 0.001; 2-tailed test; ns, not significant.
ERK1/2 stimulation since inhibition of ERK1/2 signaling by PD98059 or UO126 could impede PMA-induced PLD activation (Fig. 4A), whereas butan-1-ol (1-ButOH), a potent inhibitor of the PLD activation, did not affect ERK1/2 phosphorylation (Fig. 4C). The involvement of ERK/PLD signaling, as a potential upstream regulator of SphK1, was examined by pharmacological and genetic strategies to inhibit SphK1 activity. The treatment of PC-3 and C4-2B cells with SphK1 siRNA or SKI-II inhibitor did not interfere with PLD activity (Fig. 4A) or ERK1/2 phosphorylation (Fig. 4C). On the contrary, the pharmacological inhibition of PLD activity by 1-ButOH was associated with SphK1 activity inhibition (Fig. 4B). To rule out any possible nonspecific effect of the alcohols on SphK1 activity, the cells were treated in the presence of t-butanol (t-ButOH), a tertiary alcohol, which is not a substrate for PLD. As anticipated, unlike 1-ButOH, t-ButOH did not alter SphK1 activity (Fig. 4B). The inhibition of PLD activity by 1-ButOH triggered a loss of cell viability in both prostate cancer cell lines, which was abrogated in SphK1 overexpressing cells (Fig. 4D).

**Figure 4.** Green tea and wine polyphenols repress SphK1 activity by inhibition of ERK1/2-dependent phospholipase D activity. A) PC-3 (left panel) and C4-2B (right panel) cells were incubated with resveratrol (10 μg/ml), vineatrol (20 μg/ml), EGCg (75 μM), PPE (50 μg/ml), UO126 (10 μM), PD98059 (20 μM), and SKI (5 μM) over 15 min or with 20 nM of siSphK1 or scrambled siRNA (siScr) over 72 h. Cells were then stimulated with 100 nM PMA for 30 min, and phospholipase D activity was measured. Columns indicate means of ≥6 independent experiments; bars indicate SEM. B) PC-3 (left panel) and C4-2B (right panel) cells were treated with 1-ButOH 0.3% or t-ButOH 0.3% for 15 min and tested for SphK1 activity. Columns indicate means of 5 independent experiments; bars indicate SEM. C) PC-3 (left panel) and C4-2B (right panel) cells were incubated with SKI (5 μM), siSphK1 (20 nM), 1-ButOH (0.3%), UO126 (10 μM), or PD98059 (20 μM) for 15 min. Phosphorylation of ERK1/2 was analyzed by immunoblotting. Similar results were obtained in ≥3 independent experiments. D) PC-3/neo and PC-3/SphK1 (left panel) and C4-2B/neo and C4-2B/SphK1 (right panel) cells were treated with or without 1-ButOH 0.3% for the indicated times, and cell proliferation was assessed with 3H-thymidine incorporation assay. Columns indicate means of 6 independent experiments; bars indicate SEM. **P < 0.01, ***P < 0.001; 2-tailed test; ns, not significant.

SphK1 activity regulates the therapeutic effect of green tea and wine polyphenols in subcutaneous PC-3 tumors

We next examined whether green tea and wine polyphenols could inhibit the growth of established subcutaneous PC-3 tumors. After 2 wk of i.p. treatment with resveratrol, vineatrol, or EGCg, the average tumor volume (Fig. 5A) and intratumoral SphK1 activity (Fig. 5B) were significantly reduced as compared to untreated animals. No significant differences in the body weight of animals between controls and treated groups were noted (not shown). As SphK1 enhanced survival in PC-3 cells in response to polyphenols in vitro (Fig. 2), it was of interest to
As we previously reported in an orthotopic PC-3 xenograft model (18), we found that heterotopic PC-3/SphK1 tumors were significantly larger than those of PC-3/neo cells (Fig. 5C). The overexpression of SphK1 rendered tumors significantly less sensitive to i.p. resveratrol or EGCg treatment. Tumor size was significantly smaller by /%50 – 60% in PC-3/neo implanted tumors in animals treated with EGCg or resveratrol as compared to /%20–25% with PC-3/SphK1 cells (Fig. 5C). Accordingly, the ceramide increase and concomitant S1P decrease triggered by green tea and wine polyphenols were attenuated in animals implanted with PC-3/SphK1 (Fig. 5D).

SphK1 activity is a target of the chemopreventive effect of green tea polyphenols in orthotopic PC-3 xenografts

We next analyzed the chemopreventive efficacy of green tea polyphenols (which in contrast to wine polyphenols are easily soluble in water) using an orthotopic model of PC-3 cells overexpressing GFP, growing in their native milieu and leading to a locoregional growth and spontaneous distant metastasis dissemination (18, 19). A whole-body open imaging (not shown) of a representative untreated (drinking water) animal revealed the fluorescence of primary tumor and metastases, including periaortic and periadrenal lymph nodes, liver, pancreas, and lungs, indicating a disseminating disease (Table 1) as described previously (18, 19). Significantly smaller tumors were seen in animals given a water-based solution of 0.07% EGCg or 0.1% PPE as the unique source of dietary fluid starting the day of surgical orthotopic implantation and ending 5 wk later (not shown). This regimen was well tolerated by animals and has been used in many previous studies from other laboratories (6). Body weight of animals were unaltered by EGCg or PPE treatment (Fig. 6A). The primary tumor volume obtained by measured visualized fluorescence (Fig. 6B) may serve a reliable surrogate for tumor mass determination (Fig. 6B), both showing a strong tumor-suppressive effect of green tea polyphenols. Interestingly, the effect on primary tumor growth was paralleled by a significant limitation of metastasis occurrence in both EGCg- and PPE-treated groups (66% of animals) compared with 100% in the untreated group (Table 1). ECGg and PPE also had a remarkable effect on the total number of metastases, which was divided by 2.5–3
compared with untreated animals, respectively (Table 1). When compared to untreated animals, the number of metastases was also markedly reduced in solid organs of mice treated with EGCg or PPE, which might reflect the efficacy of both polyphenolic agents in diminishing the number (Table 1) but also the size of the remaining retroperitoneal lymph nodes (Fig. 6G). Similar to the heterotopic xenograft model, a striking inhibition (>60%) of SphK1 activity was observed in animals drinking EGCg or PPE (Fig. 6D).

**TABLE 1. Pattern of metastatic dissemination**

<table>
<thead>
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<th>Dissemination</th>
<th>Drinking water</th>
<th>EGCg</th>
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<tr>
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<td>8/12 (66%)*</td>
<td>8/12 (66%)*</td>
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<tr>
<td>Metastases</td>
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<td>18/48 (1.5/animal)*</td>
<td>18/48 (1.5/animal)*</td>
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<td>Lung</td>
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<td>0, 1, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 1</td>
<td>0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 1</td>
</tr>
<tr>
<td>Metastases</td>
<td>24/36 (2.0/animal)</td>
<td>6/36 (0.5/animal)*</td>
<td>2/36 (0.2/animal)*</td>
</tr>
<tr>
<td>Total metastases</td>
<td>57 (4.8/animal)</td>
<td>24 (2/animal)*</td>
<td>20 (1.7/animal)*</td>
</tr>
</tbody>
</table>

For retroperitoneal lymph nodes, the numbers 0, 1, or 2 represent the quantity of invaded lymph nodes. For solid organs, 0 means no presence of metastasis; 1 means a metastatic organ (regardless of the intensity of metastasis dissemination in this organ). *P = 0.0466 vs. sham-treated animals (Fisher’s exact test); †P = 0.0391 vs. sham-treated animals (Wilcoxon rank sum test); ‡P = 0.0488 vs. sham-treated animals (Wilcoxon rank sum test); ††P = 0.0039 vs. sham-treated animals (Wilcoxon rank sum test).

**Figure 6.** Effects of green tea polyphenols on growth and SphK1 activity of established human fluorescent PC-3 orthotopic xenografts in nude mice. The day of orthotopic implantation with 1 × 10^6 PC-3 cells, mice were randomized into 3 groups of 12 mice each. Animals were given drinking water, 0.07% EGCg, or 0.1% PPE solutions as the sole source of dietary fluid for 5 wk. A) Body weight was monitored every week. B) Tumor mass of excised primary tumors. C) Representative periaortic lymph nodes imaged the day of sacrifice from animals given drinking water (sham treatment; left panel) or PPE (right panel); periaortic lymph node volume calculated as described in Materials and Methods. D) SphK1 activity, S1P, and ceramide amounts were measured in tissue extracts obtained from animals the day of sacrifice. Columns indicate means from 12 animals; bars indicate SEM. *P < 0.05, **P < 0.01, ***P < 0.001; 2-tailed test; ns, not significant.
As a consequence, a substantial reduction in S1P content (Fig. 6D, middle panel) was observed in both EGCG- and PPE-treated animals. Figure 6D (right panel) shows that ceramide content was dramatically increased (~2-fold) in EGCg- or PPE-treated mice compared with untreated animals, demonstrating the shift of the ceramide/S1P biostat toward the proapoptotic ceramide.

**DISCUSSION**

The present work is the first to implicate SphK1 as a signaling target in green tea- and wine polyphenol-induced apoptosis in both hormone-responsive and hormone-refractory prostatic carcinoma cell and animal models. The SphK1/S1P pathway is associated with resistance to chemotherapy (18, 19) and radiotherapy (20), as well as the progression to hormone-refractory status (21), in prostate cancer cell and animal models. Furthermore, clinical studies conducted on prostate cancer resection specimens indicate that high expression of SphK1 directly correlates with poorly differentiated tumors (unpublished results). Here we show that SphK1 activity is rapidly and strongly inhibited in C4-2B and PC-3 cells undergoing apoptosis on treatment with green tea and wine polyphenols. In line with previous studies conducted in other cancer cell models (35, 36), PPE, which is the form of green tea used in clinical trials (see http://clinicaltrials.gov), and vineatrol extracts displayed potencies comparable to pure EGCg and resveratrol, respectively.

SphK1 activity is crucial for the growth and survival of prostate cancer cells, because its inhibition through pharmacological or silencing approaches induces apoptosis by tipping the ceramide/S1P balance toward proapoptotic ceramide (18, 19). As a proof of the critical role of SphK1, our study shows that apoptosis caused by green tea and wine polyphenols is abrogated by SphK1 overexpression, notably through inhibition of caspase-3 processing. These results are consistent with earlier reports showing that SphK1 overexpression, or addition of S1P, offers protection against proapoptotic stimuli by interfering with activation of executioner caspases (23, 37, 38). With respect to the mechanism underlying SphK1 inhibition by green tea and wine polyphenols, our results indicate that SphK1 inhibition may depend on the down-regulation of the ERK1/2 and PLD/PA signaling pathways. ERK1/2 and PLD/PA pathways have been identified as critical mediators for SphK1 mitogenic and oncogenic signaling. First, SphK1 is phosphorylated by ERK1/2, resulting in its translocation from cytosol to plasma membrane (22). Second, SphK1 activity is stimulated by PA (39), it is found up-regulated in immune cells following PLD activation (40), and more important, SphK1 binds to PA in the plasma membrane (32), where it encounters its substrate, sphingosine, to form S1P. A number of studies have also established that the PKC activator PMA stimulates SphK1 activity in an ERK1/2-dependent manner (41). PLD activity has also been widely reported to be activated by PMA (33). Here we report that green tea and wine polyphenols inhibit both PMA-stimulated PLD and ERK1/2 activities, thus resulting in SphK1 inhibition. Although resveratrol and EGCg have been reported to inhibit PMA-induced ERK1/2 activation, notably in prostate cancer (42, 43), the effect of EGCg on PMA-stimulated PLD activity has never been reported to date, whereas resveratrol was recently shown to inhibit PLD activity in neutrophils (40). Importantly, our results suggest that the inhibitory effect of polyphenols on SphK1 activity occurs through a ERK1/2-PLD signaling cascade, as the UO126 and PD98059 pharmacological inhibitors of ERK1/2 activity triggered a down-regulation of PLD activity, whereas inhibition of PLD activity by primary alcohol 1-ButOH did not affect ERK1/2 phosphorylation (Fig. 7). In agreement with a ERK1/2-PLD signaling cascade, several studies have reported that PLD activation was ERK1/2 dependent in various biological settings, including in PMA-treated colon cancer cells (44) and may rely on its phosphorylation by ERK1/2 (45). Collectively, our findings suggest that green and wine polyphenol treatment in prostate cancer is associated with an ERK1/2 inhibition followed by a PLD inhibition, leading to SphK1 inhibition. As aforementioned, our results indicate that SphK1 inhibition may depend on the down-regulation of the ERK1/2 and PLD/PA signaling pathways.

**Figure 7.** Scheme depicting the relationship between dietary polyphenols from green tea and red wine and the SphK1/S1P signaling cascade. Solid lines indicate established pathways; dotted lines indicate incomplete or still not well-defined pathways. See Discussion for more details.
tioned, both ERK1/2 and PA, the product of PLD activity, directly interact with SphK1 and regulate its biological activity (22, 32). It is hypothesized that both mechanisms may be required to obtain full activation of SphK1 (13), thus it cannot be ruled out that SphK1 inhibition initiated by polyphenols requires both direct and indirect (through PLD/PA) effect of ERK1/2.

Consistent with the curative effects of resveratrol and EGCg or PPE on the growth of implanted ovarian (46) and prostate (8) cancer cells, respectively; we show that our in vitro findings could be translated in vivo, with respect to SphK1 activity. At concentrations selected based on published effective doses in prostate and other cancer models, we demonstrate the contribution of the ceramide/S1P biotast controlled by SphK1 in response to treatment with green tea and wine polyphenols. Importantly, SphK1-overexpressing PC-3 cells subcutaneously implanted in animals developed not only remarkably larger tumors as previously reported (18) but also a resistance to resveratrol and EGCg treatment, highlighting the notion that SphK1 inhibition could be a central effector of resveratrol- and EGCg-induced apoptosis. The chemopreventive aspect with green tea or wine polyphenols has mainly been conducted in the transgenic TRAMP model, in which both resveratrol (9) and EGCg or green tea extracts (7, 47) inhibit prostate cancer progression. It is widely acknowledged that tumors grown subcutaneously are less likely to metastasize than those grown in the orthotopic site. Importantly, in conjunction with a marked inhibition of SphK1 activity and the subsequent shift of the ceramide/S1P biotast toward the proapoptotic ceramide, both EGCg and PPE diet was associated with a significant reduction in occurrence and number of metastases in the orthotopic PC-3 model. It is of interest to note that the down-regulation of SphK1/S1P signaling has been associated with lower metastatic dissemination with the chemotherapeutic agent docetaxel (18).

Increased expression of ERK1/2 (48) and SphK1 (unpublished results) are associated with prostate cancer progression in human samples. Intriguingly, the down-regulation of phospho-ERK1/2, which is a key regulator of the mitogenic and oncogenic SphK1 activity, was a significant finding in the chemopreventive studies conducted with green tea or wine polyphenols in the transgenic TRAMP model (9, 47), suggesting that ERK1/2 signaling may be a common target of polyphenolic compounds. Although no information is currently available regarding a role for SphK1 in transgenic model of the prostate carcinogenesis, it would be interesting to explore SphK1 function and assess whether SphK1 represents a target of green tea and wine polyphenols in the TRAMP mouse model of carcinogenesis. Our findings may also provide a rationale for chemoprevention in other cancer models such as colon cancer, where SphK1 is considered to play an important role in tumorigenesis (49–51). Interestingly, preventive approaches using EGCg or PPE have been shown to effectively inhibit colon carcinogenesis (52, 53). Whether SphK1 could represent a target for the development of mechanism-based chemoprevention or therapeutic strategies against colon cancer development remains to be investigated.

With respect to prostate cancer therapy, it may be efficacious to combine green tea or wine polyphenols with chemotherapeutic agents or radiation therapy. First, we have previously reported that pharmacological inhibitors of SphK1 exert synergistic growth inhibition of prostate cancer cells in vitro and in animal models, when combined with chemotherapy (18, 19, 54). Second, we recently identified SphK1/S1P pathway as a new modulator of the transcription factor HIF-1α, the master regulator of the adaptation to hypoxia (27), driving tumor progression and impairing the effectiveness of chemotherapy and radiotherapy. By inhibiting hypoxia and its molecular consequences, inhibition of the SphK1/S1P signaling has been postulated to increase tumor sensitivity to therapy (55). Hypoxic regions exist in human prostate carcinoma, and increasing levels of hypoxia are associated with higher clinical stages. Interestingly, both resveratrol and EGCg have been shown to inhibit hypoxia-induced accumulation of HIF-1α in other cancer cell models (56, 57). Further investigations are requested to validate a role for green tea and wine polyphenols as sensitizers, through SphK1-mediated inhibition of hypoxia, for the current prostate cancer therapeutic arsenal.

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