Sphingosine-1-Phosphate Inhibits Nuclear Factor κ B Activation and Germ Cell Apoptosis in the Human Testis Independently of Its Receptors

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Early apoptosis-inducing events are potentially important targets for preventing germ cell loss caused by external stress. The sphingolipid sphingosine-1-phosphate (S1P) is an important regulator of stress-induced apoptosis. It affects the cell as an intracellular signaling molecule or as a ligand to its cell membrane-bound S1P₁₋₅ receptors. We previously demonstrated that S1P inhibits stress-induced male germ cell death *in vitro* and *in vivo*. Here, we further define the mechanisms of S1P-mediated inhibition of male germ cell death. Using immunohistochemistry, we detected expression of the S1P₁ and S1P₂ receptors in the somatic Sertoli cells of the human testis. In a culture of human seminiferous tubules, S1P inhibited germ cell apoptosis, suppressed both nuclear factor κ B (NF- κ B) DNA-binding activity and expression of phosphorylated Akt, but did not affect activator protein-1 (AP-1) DNA-binding activity. Dihydro-S1P, which binds to and activates S1P receptors but has no direct intracellular effect, suppressed neither apoptosis nor NF- κ B activity. These results suggest that S1P inhibits male germ cell apoptosis independently of its receptors, possibly by inhibiting the transcription factor NF- κ B and Akt phosphorylation. (*Am J Pathol* 2005, 166:773–781)

Sphingolipids are cell membrane phospholipids that are widely distributed in mammalian cells.¹ Beyond their role in structural organization, they have diverse functions, such as regulation of cell growth, differentiation, and apoptosis.² Ceramide, the structural backbone of the sphingolipids, is formed from cell membrane sphingomyelin by sphingomyelinases, which are activated by a

variety of stress factors such as anticancer drugs, ionizing radiation, tumor necrosis factor α (TNF α), Fas ligand, growth factor withdrawal, and oxidative stress.^{3,4} Ceramide acts as a mediator of cell-growth arrest and apoptosis in many tissues and cell lines.^{3,5} Sphingosine-1-phosphate (S1P), synthesized from sphingosine by sphingosine kinase (SPHK), inhibits apoptosis that results from elevated levels of ceramide⁶ and regulates a variety of proliferative cellular processes including cell growth and differentiation.^{7–9}

S1P exerts its effects either by acting as an intracellular signaling molecule or via its specific, cell membrane-bound S1P₁₋₅ receptors (formerly EDG -1, -3, -5, -6, and -8 receptors), which couple to the G_i, G_q, or G_{12/13} types of G proteins.¹⁰ Initially, it was suggested that S1P acts as an intracellular signaling molecule and the balance between intracellular levels of S1P and ceramide is crucial to determining whether the cell will survive or die.¹¹ The majority of the recent studies, however, have implicated an important role of G protein-coupled S1P receptors in mediating biological functions of S1P. These receptors integrate signals with specific signal transduction pathways, eg, activation of the extracellular signal-regulated kinase (ERK1/2), the p-38 kinase, and the c-Jun NH2-terminal kinase (JNK) pathways.^{12–14} Receptor activation also leads to other intracellular changes, such as decreased levels of cyclic AMP (cAMP),⁹ and increased production of inositol phosphate and level of Ca²⁺.¹⁵ The targets of the S1P-induced intracellular signaling, however, remain unclear, but the transcription factors nuclear factor kappa B (NF- κ B)¹⁶ and activator protein-1 (AP-1)¹⁷ may be involved.

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Recently we demonstrated that the level of testicular ceramide increases rapidly during *in vitro*-induced apoptosis of human male germ cells, which likely results from the breakdown of sphingomyelin by sphingomyelinases. Consistently, germ cell apoptosis can be suppressed by exogenous S1P.¹⁸ In addition, we observed that in mice, intratesticular S1P treatment moderately prevents irradiation-induced male germ cell apoptosis *in vivo*.¹⁹ The aim of the present study was thus to characterize the mechanisms by which S1P inhibits human male germ cell apoptosis. Since we previously found that NF- κ B²⁰ and AP-1²¹ are activated during the induction of human male germ cell death, we evaluated whether the regulation of these transcription factors is involved in the cell death-inhibiting effect of S1P.

Patients and Methods

Patients

Testicular tissue was obtained from seven men, ranging in age from 59 to 78 undergoing orchidectomy as a treatment for prostate cancer. These patients had received no hormonal, chemotherapeutic, or radiotherapeutic treatment before the operations. They had no endocrinological disease and none had suffered from cryptorchidism. The testicular tissue was prepared for culture immediately after the operations, which were performed between January 2003 and August 2003 at the department of Urology, Helsinki University Central Hospital (Helsinki, Finland). The ethics committees of the Hospital for Children and Adolescents and the Department of Urology, University of Helsinki, approved the study protocol.

Tissue Culture

Apoptosis of the human testicular germ cells was induced by incubating segments of seminiferous tubules under serum-free conditions. Physiological contact between the Sertoli cells and the germ cells was maintained by culturing these tubule segments rather than isolated testicular cells. The testicular tissue was microdissected on a petri dish containing culture medium (nutrient mixture Ham's F10; Gibco, Europe) supplemented with 0.01% human serum albumin (Sigma Chemical Co., St. Louis, MO) and 10 μ g/ml gentamicin (Gibco). The tubule segments were transferred to a Petri dish containing the same serum-free culture medium and incubated for 0 to 24 hours at 34°C in a humidified atmosphere containing 5% CO₂.

Southern Blot Analysis of Low-Molecular Weight DNA Fragmentation

DNA was extracted with the apoptotic DNA Ladder kit (Roche Molecular Biochemicals, Mannheim, Germany) as described.²² DNA was quantified spectrophotometrically (absorbance at 260 nm), and 1 μ g of the total DNA

from each sample was subjected to 3'-end-labeling with digoxigenin-dideoxy-UTP (dig-dd-UTP, Roche) using the terminal transferase reaction (Roche). The DNA samples were electrophoresed on 2% agarose gels, blotted onto nylon membranes, and cross-linked to the membranes with UV irradiation. The membranes were then washed and blocked with 1% blocking reagent (Roche) in maleic acid buffer (100 mmol/L maleic acid, 150 mmol/L NaCl, pH 7.5) for 30 minutes at room temperature. The 3' end-labeled DNA in the membranes was localized with alkaline phosphatase-conjugated anti-digoxigenin antibody (Anti-Digoxigenin-AP; Roche), and the bound antibody was detected using the chemiluminescence reaction (CSPD; Roche). The X-ray films exposed to chemiluminescence were scanned with a tabletop scanner (Hewlett-Packard Scanjet 6300 C), and the digital image was analyzed with the Gel Plot 2 macro for Scion image β 4.0.2. (Scion Corporation, Frederick, MD) analysis software. The amounts of apoptotic low-molecular weight DNA fragments (<1.3 kB) in various samples were expressed in relation to low-molecular weight DNA in the sample cultured for 5 hours in the serum-free medium without treatments, which was taken as 1.0 (100% apoptosis).

Immunohistochemistry

Immunostainings of EDG-1 (S1P₁), EDG-5 (S1P₂), phosphorylated ERK, and ERK were performed on paraffin-embedded sections of formalin-fixed adult human testis tissues. The paraffin sections were incubated at 60°C for 30 minutes and deparaffinized in xylene. The sections were then rehydrated, microwaved at high power for 5 minutes in citrate buffer (10 mmol/L citrate, pH 6.0) for antigen retrieval, washed, and blocked with blocking solution [phosphate-buffered saline (PBS) containing 5% goat or donkey normal serum, 3% bovine serum albumin, and 0.1% Tween] for at least 30 minutes at room temperature. The EDG-1 and EDG-5 proteins were detected using goat polyclonal antibodies to human EDG-1 and EDG-5 (sc-16070 and sc-16085; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The phosphorylated ie, active forms of the ERK1/2 [p-p44/42 MAP kinase (MAPK)] and inactive ERK were detected using rabbit polyclonal antibodies against human p-ERK (MAPK detection kit, 9910; Cell Signaling Technology Inc., Beverly, MA) and ERK (sc-94; Santa Cruz). The antibodies were used at concentrations of 0.5 to 0.7 μ g/ml. The primary antibodies in blocking solution were added to the samples, which were then incubated overnight at 4°C. After incubation, the slides were washed with PBS. The primary antibodies were detected using biotin-conjugated donkey anti-goat, goat anti-rabbit, or rabbit anti-mouse IgGs from the corresponding ABC-Elite kits (Vector Laboratories, Inc., Burlingame, CA) followed by incubation with the ABC solution. For location of the secondary antibody, 0.05% diaminobenzidine substrate (Sigma) was added. After the staining protocols, light counterstaining was performed with hematoxylin and the samples were dehydrated and mounted. For the negative controls, the primary antibodies were replaced with nonimmune rabbit or

mouse IgG (Sigma). Blocking peptides were used to verify the specificity of the polyclonal antibodies to EDG-1 and EDG-5 (sc-16070 P and sc-16085 P; Santa Cruz).

Protein Extracts

For cytoplasmic and nuclear protein extracts, seminiferous tubules were gently homogenized with a tight-fitting Potter-Elvehjem homogenizer in ice-cold hypotonic buffer A [50 mmol/L HEPES (pH 7.4), 10 mmol/L KCl, 1 mmol/L ethylenediaminetetra-acetic acid, 1 mmol/L dithiothreitol, 0.3 mmol/L phenylmethylsulfonyl fluoride, 1 μ g/ml pepstatin A, 1 μ g/ml leupeptin, 0.5% Nonidet P-40], and the protein extracts were prepared as previously described.²² For whole cell protein extracts, small tissue sections were homogenized with an Ultra-Turrax T8 homogenizer on ice in homogenization buffer [1% Triton X-100, 150 mmol/L Tris (pH 7.4), 1 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L ethyleneglycol-bis-(β -aminoethylether)-N'-N'-N'-tetraacetic acid, 0.2 mmol/L phenylmethylsulfonyl fluoride, and 1 μ g/ml leupeptin]. After centrifugation at $17,000 \times g$ for 30 minutes, the supernatants containing the whole-cell proteins were collected. The protein concentrations were determined using the DC protein assay (Bio-Rad Laboratories, Inc., Hercules CA) and the protein extracts were stored at -80°C until used in Western blotting or electrophoretic mobility shift assays (EMSAs).

Electrophoretic Mobility Shift Assay

The NF- κ B and AP-1 DNA-binding activities were assayed with DNA probes containing the consensus κ B enhancer element 5'AGTTGAGGGGACTTTCCCAGGC-3'(sc-2505; Santa Cruz) or the consensus AP-1 site 5'GATCTATCTGAGTCAGCAG-3'.²³ The probes were 5'-end-labeled with [γ -³²P] ATP using polynucleotide kinase (Promega Corp., Madison, WI). Nuclear protein extracts (10 μ g) were incubated on ice for 10 minutes with 2 μ g poly(dIdC)(di-dC) (Amersham Pharmacia Biotech, Piscataway, NJ) in 50 mmol/L HEPES, (pH 7.6), 10% glycerol v/v, 225 mmol/L KCl, 1 mmol/L ethylenediaminetetraacetic acid, 2.5 mmol/L dithiothreitol, 1 mmol/L MgCl₂, 0.75 mmol/L phenylmethylsulfonyl fluoride, and 1.5 μ mol/L leupeptin. A 5' end-labeled probe (15,000 to 30,000 cpm) was then added and incubation was continued at room temperature for 30 minutes. In the competition experiments, a 100-fold molar excess of unlabeled probe or unlabeled mutated probe (Santa Cruz; sc-2511) was added before the labeled probe. The reaction products were separated on 4% polyacrylamide gels run in 22.5 mmol/L Tris-borate and 0.5 mmol/L ethylenediaminetetraacetic acid at 200 V at room temperature. After electrophoresis, the gels were dried and visualized with autoradiography.

Western Blotting

Western blotting of inhibitory kappa B (I κ B α) (sc-847; Santa Cruz) was performed using cytoplasmic protein extracts of seminiferous tubules. Western blotting of p-

Akt (9271, Cell Signaling Technology) were performed from whole-cell protein extracts of seminiferous tubules.

The proteins (50 μ g) were loaded into 10% sodium dodecyl sulfate-polyacrylamide gels and electrophoresis was performed at 180 V. The proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp., Bedford, MA) by electrophoresis for 2 hours at 4°C in transfer buffer (26 mmol/L Tris, 192 mmol/L glycine, 10% methanol) at 100 V. The transfer was checked by staining with 0.2% Ponceau S in 3% trichloroacetic acid. The primary antibodies against the proteins under investigation were used at 0.2 μ g/ml and were followed with peroxidase-conjugated goat anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA) or peroxidase-conjugated goat anti-mouse (DAKO Corp., Glostrup, Denmark) IgG. The bound secondary antibodies were located with ECL detection kit (Amersham, Arlington Heights, IL). After detection of the proteins under investigation, the membranes were washed and, as a loading control, probed with an antibody to α -tubulin (Sigma; loading control to I κ B α and p-JNK) or Akt (9272; Cell Signaling Technology; loading control to p-Akt).

To examine the alterations in protein expressions of I κ B α and p-Akt, the X-ray films exposed to ECL were scanned and the digital images were analyzed with Scion Image β 4.0.2. analysis software (Scion Corp.). Standard curves for I κ B α , α -tubulin, p-Akt, and Akt were constructed with a dilution series of a control sample. The amounts of I κ B α or p-Akt in the samples were adjusted to the amount of their loading controls ie, α -tubulin and Akt, respectively, in the corresponding samples.

Treatments

The effects of S1P and dihydro-S1P (an analog of S1P that activates S1P receptors, but has no direct intracellular effects of S1P) on human male germ cell apoptosis, and on NF- κ B and AP-1 DNA-binding activities were studied by adding S1P or dS1P (Biomol Research Laboratories, Inc., Plymouth, MA) to the culture medium and by determining the amount of low-molecular weight DNA fragmentation and NF- κ B and AP-1 DNA-binding activities in seminiferous tubules cultured for 5 hours in the absence or presence of S1P or dS1P. For the experiments, S1P and dS1P were first dissolved in methanol (0.5 mg/ml). The methanol stock was aliquoted and the solvent evaporated with a nitrogen stream. Immediately before use, the S1P and dS1P were dissolved in the culture medium to prepare a 125 μ mol/L stock and used at final concentrations of 10 μ mol/L. In our preliminary experiments, dS1P was also used at concentrations of 1 μ mol/L and 20 μ mol/L (data not shown).

Statistics

The cultures used for studying the effects of S1P and dS1P on low-molecular weight DNA fragmentation were repeated on five independent occasions. The quantitative data represent integrated optical densities from scanned X-ray films. Data obtained from five cultures

Figure 1. Expression of the S1P₁ receptor (EDG-1) and S1P₂ receptor (EDG-5) in the human testis. Paraffin-embedded sections of formalin-fixed adult human seminiferous tubules were immunostained with antibodies against EDG-1 and EDG-5. **a** and **c**: All of the cross sections of the seminiferous tubules showed positive immunostaining for EDG-1 and EDG-5. The staining pattern was typical for Sertoli cell cytoplasm. **b** and **d**: When the antibodies were replaced with specific blocking peptides (BP), no specific immunostaining was observed. Original magnification, $\times 200$. **e** and **f**: Original magnification of $\times 1000$ demonstrating that the staining was cytoplasmic and was not present in the germ cells.

(mean $\pm$ SEM) were analyzed with one-way analysis of variance, which (if significant differences existed) was followed by comparison of the groups with the two-tailed unpaired Student's *t*-test, in which a value of $P < 0.05$ was considered statistically significant. The EMSAs used for studying the effect of S1P or dS1P on NF- κ B DNA binding activity were performed using samples from three independent cultures and those for studying the effects of S1P or dS1P on AP-1 DNA-binding activity were performed using samples from two independent cultures. The immunohistochemical analysis with each antibody was performed from three independent tissues and on at least three parallel slides. The effects of S1P and dS1P on the expressions of I κ B α / α -tubulin and p-Akt/Akt were studied with Western blotting using samples from three independent cultures.

Results

Expressions of S1P₁ (EDG-1) and S1P₂ (EDG-5) Receptors in the Human Testis

In paraffin-embedded sections of formalin-fixed, non-cultured human seminiferous tubules, both the antibodies against the S1P₁ (EDG-1) and S1P₂ (EDG-5) receptors gave similar cytoplasmic staining pattern: positive immunostaining was observed in the Sertoli cells in the majority of the seminiferous tubule cross sections (Figure 1). The positively stained cells were identified as Sertoli cells based on their basal location in the seminiferous tubules and the characteristic morphology of their cytoplasm.

Occasional germ cells, mainly spermatogonia and early meiotic spermatocytes, also expressed weak cytoplasmic immunostaining. When the primary antibodies were replaced with blocking peptide specific to the antibodies, no specific immunostaining was detected (Figure 1).

S1P Inhibits Human Male Germ Cell Apoptosis Independently of the S1P Receptors

In the present *in vitro* culture model, segments of the seminiferous tubules rather than isolated germ cells were cultured to maintain the germ cells in their physiological environment. Culturing the tubule segments under serum-free conditions resulted in strong induction of apoptotic DNA fragmentation within 5 hours (Figure 2). In previous studies we identified the cell types undergoing apoptosis most frequently as spermatocytes and spermatids, using *in situ* 3'-end-labeling (ISEL)²⁴ and electron microscopy.²⁵ Consistent with our previous findings by Southern blot and ISEL analyses,¹⁸ S1P at a concentration of 10 μ mol/L inhibited apoptosis by 28% ($P < 0.05$; Figure 2).

Unlike S1P, dS1P did not have an inhibitory effect on apoptotic DNA fragmentation. The amount of DNA fragmentation in the tubules treated with 10 μ mol/L dS1P was 108% compared with the non-treated, 5 hours cultured samples ($P = \text{NS}$; Figure 2). The concentrations of 1 μ mol/L and 20 μ mol/L of dS1P likewise had no effects on germ cell apoptosis (data not shown).

Figure 2. Regulation of *in vitro*-induced human male germ cell apoptosis by S1P and dS1P. Segments of seminiferous tubules were incubated under serum-free conditions for 5 hours in the presence or absence of 10 μ mol/L S1P or dS1P. DNA from the seminiferous tubules was extracted, after which equal amounts (1 μ g) of the total DNA from each sample was 3'-end-labeled with Dig-dd-UTP. The DNA samples were electrophoresed and blotted onto nylon membranes and the labeled apoptotic DNA fragments were detected with chemiluminescence as described in the Materials and Methods section. **a:** Radiograph from a representative experiment, in which S1P or dS1P was added to the culture medium at 10 μ mol/L. **b:** Quantification of low-molecular weight (MW) DNA fragmentation. Each value represents the mean of 5 ($n = 5$) independent experiments $\pm$ SEM; * $P < 0.001$, NS = not significant.

S1P but Not dS1P Inhibits NF- κ B DNA-Binding Activity and Phosphorylation of Akt

According to our previous findings,²⁰ EMSA of nuclear protein extracts from seminiferous tubules cultured for 5 hours under serum-free conditions without treatments revealed three NF- κ B-specific bands. Nearly complete suppression of this NF- κ B DNA-binding activity was observed in the seminiferous tubules cultured for 5 hours

Figure 3. Effects of S1P and dS1P on NF- κ B and AP-1 DNA-binding activities. Segments of human seminiferous tubules were cultured in the absence or presence of 10 μ mol/L S1P or dS1P. After the 0-hour and 5-hour culture, the samples were snap-frozen for nuclear protein extracts and NF- κ B and AP-1 DNA binding activities were analyzed. **A:** EMSA showing the effects of 10 μ mol/L S1P and dS1P on testicular NF- κ B DNA-binding activity. Nuclear protein extracts (10 μ g) from the seminiferous tubules were analyzed for the presence of NF- κ B DNA-binding activity ($n = 3$). Three NF- κ B-specific bands were observed (A, B, and C). Competitive experiment using unlabeled (cold) κ B-oligonucleotide or unlabeled, mutated κ B oligonucleotide (mut.) confirmed the specificity of the NF- κ B complex. The result is representative of three independent cultures. EMSA showing the effects of 10 μ mol/L S1P and dS1P on testicular AP-1 DNA-binding activity. Nuclear protein extracts (10 μ g) from the seminiferous tubules were analyzed for AP-1 DNA-binding activity ($n = 2$) as described. Competitive experiment using unlabeled (cold) AP-1 oligonucleotide confirmed the specificity of the AP-1 complex. NS = not specific. The result is representative of two independent cultures.

Figure 4. Effects of S1P and dS1P on I κ B degradation and expression of p-Akt. **A:** Western blot analysis of I κ B α . Cytosolic protein extracts were prepared from human seminiferous tubules cultured for 0 or 5 hours in the absence or presence of S1P or dS1P. Equal amounts of protein were subjected to electrophoresis, electroblotted, and probed with a rabbit polyclonal antibody to human I κ B α . α -tubulin was used as a loading control. **A, b:** Quantification of I κ B α expression (adjusted to the amount of α -tubulin) from Western blots of three independent cultures. In each culture, I κ B α was more strongly expressed in S1P-treated tubules than in the non-treated or dS1P-treated tubules. **B, a:** Western blot from whole cell protein extracts demonstrating the effects of S1P and dS1P on the testicular expression of p-Akt was performed as described. Inactive Akt was used as a loading control. **A, b:** Quantification of p-Akt expression (adjusted to the amount of total Akt) from Western blots of three independent cultures. In each culture, the expression of p-Akt was clearly diminished in S1P-treated tubules compared with its expression in non-treated or dS1P-treated tubules.

with 10 μ mol/L S1P ($n = 3$) (Figure 3A). In contrast, no effect on NF- κ B DNA-binding activity could be seen compared with the non-treated samples in the tubules cultured for 5 hours with 10 μ mol/L dS1P ($n = 3$) (Figure 3A). Competition experiments with untreated, 5-hour-cultured tubules using unlabeled (cold) or unlabeled mutated κ B oligonucleotides confirmed the binding specificity of the κ B oligonucleotide (Figure 3A). The AP-1 DNA-binding activity was also increased in the seminiferous tubules cultured for 5 hours under serum-free conditions. Neither S1P nor dS1P affected this AP-1 activation (Figure 3B).

Western blot analysis of I κ B α showed intensive I κ B α expression in the uncultured (0 hours) seminiferous tubules and clearly diminished I κ B α expression in the tubules cultured for 5 hours without treatments (Figure 4A). In the tubules treated with 10 μ mol/L S1P ($n = 3$), I κ B α was more strongly expressed than in the non-treated tubules in two of the three replicate cultures (Figure 4A). Equal loading of the samples was confirmed by reprob- ing the blots with an antibody against α -tubulin.

Western blot analysis of Akt ($n = 3$) showed that p-Akt was strongly expressed in the uncultured seminiferous tubules (0 hours), whereas in the 5-hour-cultured tubules, its expression was clearly diminished (Figure 4B). In the S1P-treated 5-hour-cultured tubules, the expression of p-Akt was almost totally absent. In contrast, expression of

p-ERK

0 hours

5 hours

5 hours

5 hours

ERK

Figure 5. Cellular expression of p-ERK and ERK during *in vitro*-induced human testicular apoptosis. Immunostaining for the p-ERK and ERK were performed in paraffin-embedded sections of formalin-fixed human seminiferous tubules cultured for 0 or 5 hours under serum-free conditions. In non-cultured samples (0 hours), weak immunostaining for the p-ERK was observed in the nuclei and cytoplasm of the Sertoli cells and in some primary spermatocytes. After the 5-hour culture, some segments of the seminiferous tubules showed nuclear and cytoplasmic staining for the p-ERK antibody. In the S1P-treated, 5-hour-cultured samples, nuclear and cytoplasmic immunostaining of the Sertoli cells and some primary spermatocytes were clear in all of the seminiferous tubules detected. In the dS1P-treated 5-hour-cultured samples, the intensity of the immunostaining was further increased. Immunostaining for the ERK was similar in 5-hour-cultured, non-treated, S1P-treated, and dS1P-treated samples, it was strongly expressed in both the Sertoli cells and the germ cells. When the antibodies were replaced with non-specific rabbit IgG (Neg), no specific immunostaining was observed. Original magnification, $\times 200$.

p-Akt was evident in the dS1P-treated 5-hour-cultured samples (Figure 4B). Equal amounts of inactive Akt, which served as a loading control, were detected in the uncultured, 5-hour-cultured untreated, and 5-hour-cultured, S1P- or dS1P-treated seminiferous tubules (Figure 4B).

S1P Induces Sertoli Cell ERK Activation

The expression of p-ERK and ERK were studied with immunohistochemistry in paraffin-embedded sections of formalin-fixed human seminiferous tubules cultured for 0 hours or 5 hours in the presence or absence of $10 \mu\text{mol/L}$ S1P or dS1P. In uncultured seminiferous tubules, some Sertoli cells expressed weak cytoplasmic immunostaining. After a 5-hour culture in the presence or absence of S1P or dS1P, the Sertoli cells and some primary spermatocytes in most of the seminiferous tubules expressed cytoplasmic and nuclear p-ERK (Figure 5). However, positive staining was more intense in the tubules treated

with S1P or dS1P and most prominent in the tubules treated with dS1P. The expression of ERK representing overall levels of ERK was similar in the samples cultured for 5 hours with or without treatments; strong expression of ERK was found in the cytoplasm of both the germ cells and the Sertoli cells (Figure 5). When the primary antibody was replaced with similar concentration of non-specific rabbit IgG, no specific immunostaining could be detected (Figure 5).

Discussion

During spermatogenesis, the proliferation and differentiation of spermatogonia into spermatozoa through several mitotic and meiotic divisions, the number of maturing germ cells is regulated by apoptosis to match the capacity of the supporting nonproliferative somatic Sertoli cells.^{26–28} This physiological germ cell death is a prereq-

uisite for normal spermatogenesis.²⁹ However, inadequate germ cell death, which may result from external stress such as chemotherapy or irradiation, leads to spermatogenic arrest and even infertility.^{30,31} We previously demonstrated that S1P is able to inhibit stress-induced male germ cell apoptosis *in vitro* and *in vivo*.^{18,19} Here, we clarify the mechanisms of S1P-mediated inhibition of human male germ cell death. Consistent with previous findings in the rodent testis,¹⁰ we detected the expression of the S1P₁ (EDG-1) and S1P₂ (EDG-5) receptors in the somatic Sertoli cells. Interestingly, S1P inhibited human testicular apoptosis independently of its receptors, possibly by inhibiting transcription factor NF- κ B.

Several findings support the role of S1P as an intracellular second messenger.² We found that dS1P, an analog of S1P that binds to and activates all S1P receptors but does not act as an intracellular second messenger *per se*, was not able to inhibit human male germ cell death, indicating that this inhibitory effect of S1P is independent of its receptors expressed in the Sertoli cells. This finding is supported by similar data on several cell lines, in which dS1P was not able to mimic all of the effects of S1P, especially those related to cell survival.^{9,32,33} Moreover, our results show that NF- κ B activity may be regulated by S1P independently of the S1P receptors, since dS1P did not affect NF- κ B DNA-binding activity. We previously showed that NF- κ B is activated rapidly in Sertoli cells and that the anti-inflammatory drug sulfasalazine almost totally blocked both germ cell apoptosis and Sertoli cell NF- κ B DNA-binding activity possibly by preventing the NF- κ B-induced synthesis of new I κ B α protein.²⁰ These results suggest that the inducible NF- κ B in the Sertoli cells may regulate Sertoli cell genes that participate in the control of germ cell death during the induction of human male germ cell apoptosis. That S1P inhibits both apoptosis and NF- κ B activation supports the role played by Sertoli cell NF- κ B in the induction of germ cell death. Somewhat surprisingly, S1P effectively inhibited the Sertoli cell NF- κ B DNA-binding activity, whereas it inhibited male germ cell apoptosis only by 28%. One explanation for this discrepancy is that Sertoli cell NF- κ B may not play a central role in induction of male germ apoptosis. More likely, possibly by binding to its receptors in Sertoli cells, S1P may induce parallel pro-apoptotic pathways simultaneously with NF- κ B inhibition.

NF- κ B is a transcription factor that regulates a variety of genes. Before activation, NF- κ B proteins are sequestered in the cytosol by the inhibitory I κ B proteins that release NF- κ B after degradation, allowing its translocation into the nucleus.³⁴ We previously showed that in the present culture model, the best characterized I κ B protein, I κ B α , is degraded in the seminiferous tubules during germ cell apoptosis.²⁰ Here, inhibition of NF- κ B DNA-binding activity by S1P was associated with suppression of I κ B α degradation, suggesting that S1P regulates NF- κ B activity by inhibiting degradation of I κ B. The degradation of I κ B is controlled by the I κ B kinase (IKK) complex, which in turn is regulated by several mechanisms, including the PI3/Akt cascade.³⁵ The PI3/Akt kinase cascade regulates multiple cellular processes, and its activation is usually related to cell survival.³⁵ Activation

Figure 6. Schematic illustration demonstrating hypothetical inhibitory pathway of S1P on germ cell apoptosis of the human testis. To inhibit male germ cell apoptosis, S1P suppresses activation of NF- κ B upstream by inhibiting the phosphorylation of Akt and the degradation of I κ B α . S1P is generated intracellularly by phosphorylation of sphingosine (which is degraded from ceramide by ceramidase) by the enzyme SPHK. Intracellularly generated S1P inhibits phosphorylation of Akt, which results in inhibition of IKK. IKK, in turn, regulates degradation of I κ B α , which sequesters NF- κ B in the cytosol. Since I κ B α degradation is suppressed due to the inhibition of IKK, entry of NF- κ B into the nucleus, and thus male germ cell apoptosis, is inhibited. S1P receptors (S1PR) do not appear to play a role in the regulation of male germ cell apoptosis.

of Akt results in the phosphorylation of a number of substrates including IKK, Bad, caspase 9, forkhead transcription factors, and endogenous nitric oxide synthase.³⁵ Phosphorylation of these proteins by Akt results in their activation or inactivation, depending on the substrate. Since Akt may regulate NF- κ B via the IKK-I κ B regulatory cascade, we wished to determine whether this cascade was involved in the S1P-mediated inhibition of human male germ cell death. We found that S1P inhibits the phosphorylation of Akt and the degradation of I κ B α concomitantly with the inhibition of NF- κ B activation and apoptosis. In contrast, dS1P showed no inhibitory effect on these processes. Thus, our results suggest that during inhibition of male germ cell apoptosis, S1P suppresses NF- κ B activation upstream by acting as an intracellular second messenger that inhibits the phosphorylation of Akt, which in turn inhibits the degradation of I κ B proteins (possibly by inhibiting IKK), finally resulting in inhibition of NF- κ B activation (Figure 6). Cross talk between the sphingomyelin- and PI3/Akt pathways as a mechanism for cell survival/death decisions has indeed been suggested.^{36,37} The finding that the inhibition of apoptosis by S1P but not dS1P is associated with the inhibition of Akt is interesting, since strong evidence exists for S1P promoting cell survival via binding to its receptors and activating

the PI3/Akt kinase cascade.^{10,12,38,39} In keratinocytes, however, S1P plays an important role in the regulation of proliferation by inhibiting Akt independently of S1P receptors.⁴⁰ Thus, whether S1P regulates Akt activation via the S1P receptors or by acting as an intracellular second messenger, appears to be highly dependent on the surrounding conditions and the cell.

We recently observed that the transcription factor AP-1 is also activated during human male germ cell apoptosis.²¹ AP-1 is a collective term referring to dimeric transcription factor proteins belonging to the Jun, Fos, and ATF subfamilies.⁴¹ Activity of AP-1 is induced by multiple environmental insults and physiological stimuli that activate mitogen activated protein kinases ERK1/2, JNK, and the p38 enzymes.⁴² Here, we demonstrated that neither S1P nor dS1P affected AP-1 DNA-binding activity, suggesting that AP-1 is not involved in the S1P-mediated inhibition of human male germ cell apoptosis. Both the intracellular second messenger and extracellular receptor-mediated signaling pathways of S1P have been suggested to inhibit apoptosis via activation of ERK.^{2,10} We found increased expression of p-ERK in the Sertoli cells and some spermatocytes both in S1P- and dS1P-treated seminiferous tubules, the expression being greater in the latter. This suggests that the function(s) of ERK in the present model are related to S1P receptors, but may play roles other than those involved in the S1P-mediated inhibition of the germ cell death.

In conclusion, we explored the mechanisms of S1P-mediated germ cell survival to better understand its potential role in the inhibition of stress-induced human male germ cell death. Our data suggest that the S1P receptors are not involved in the inhibition of germ cell apoptosis and that suppression of apoptosis by S1P is associated with inhibition of NF- κ B and p-Akt, but not AP-1 or the MAPKs. These results may aid future attempts to protect male gonads from apoptosis and subsequent infertility caused by external stress, such as cancer therapy.

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