Dynamic regulation of sphingosine-1-phosphate homeostasis during development of the mouse metanephric kidney

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Running head: Sphingosine-1-phosphate and kidney development

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ABSTRACT

Branching morphogenesis of the metanephric kidney is critically dependent on the delicate orchestration of diverse cellular processes including proliferation, apoptosis, migration, and differentiation. Sphingosine-1-phosphate (S1P) is a potent lipid mediator influencing many of these cellular events. We report increased expression and activity of both sphingosine kinases and S1P phosphatases during development of the mouse metanephric kidney from induction at E11.5 to maturity. Sphingosine kinase activity exceeded S1P phosphatase activity in embryonic kidneys resulting in a net accumulation of S1P, while kinase and phosphatase activities were similar in adult tissue resulting in reduced S1P content. Sphingosine kinase expression was greater in the metanephric mesenchyme than in the ureteric bud while the S1P phosphatase, SPP2, was expressed at greater levels in the ureteric bud. Treatment of cultured embryonic kidneys with sphingosine kinase inhibitors resulted in a dose-dependent reduction of ureteric bud tip numbers and increased apoptosis. Exogenous S1P rescued kidneys from apoptosis induced by kinase inhibitors. Ureteric bud tip number was unaffected by exogenous S1P in kidneys treated with N,N-dimethylsphingosine, though tip number increased in those treated with D,L-threo-dihydrosphingosine. S1P<sub>1</sub> and S1P<sub>2</sub> were the predominant S1P receptors expressed in the embryonic kidney. S1P<sub>1</sub> expression increased during renal development while expression of S1P<sub>2</sub> decreased, and both receptors were expressed predominantly in the metanephric mesenchyme. These results demonstrate dynamic regulation of S1P homeostasis during renal morphogenesis, and suggest that differential expression of S1P metabolic enzymes and receptors provide a novel mechanism contributing to the regulation of kidney development.
Key words: renal morphogenesis, sphingolipid, kinase, phosphatase, receptor
INTRODUCTION

Development of the metanephric kidney is a highly complex event orchestrated by numerous factors regulating proliferation, migration, and differentiation of individual cell types to form the functional kidney. This process is initiated by induction of the ureteric bud, an outgrowth of the Wolffian duct, by the metanephric mesenchyme. Subsequent branching of the ureteric bud induces mesenchymal-to-epithelial transformation and nephron formation. Inductive interactions between the ureteric bud epithelia and the metanephric mesenchyme are mediated by a multitude of stimulatory and inhibitory factors (7, 42). Failure of mechanisms regulating normal kidney development can result in a variety of congenital defects and may contribute to nephron deficiencies leading to development of hypertension and renal failure.

Sphingosine-1-phosphate (S1P) is a potent bioactive lipid that functions both as an intracellular second messenger promoting cell survival and proliferation, and as a ligand for G-protein-coupled cell surface receptors (S1PRs) to influence migration and differentiation. S1P has a powerful influence on developmental processes and recent studies have clearly demonstrated that S1P signaling is essential to the development of vascular and neural systems as well as morphogenesis of the heart and pancreas (3, 9, 31, 49). The role of S1P in regulation of kidney development, however, has not been examined.

Within the cell, S1P is in dynamic balance with its precursors, ceramide and sphingosine, forming what is termed the ‘sphingolipid rheostat’ (43). Ceramide has an opposing action to S1P, inhibiting growth and promoting apoptosis (39). The balance between S1P and ceramide therefore determines whether cells undergo apoptosis or
proliferation. Secreted S1P further provides autocrine and paracrine signals through activation of five known S1PRs (S1P1-5). S1PR activation influences a variety of downstream signaling pathways including Ca\(^{2+}\) mobilization, cAMP regulation, inositol phosphate metabolism, activation of PI3K, Akt, ERK, and phospholipase C (15). S1P concentration is a primary determinant of all subsequent signaling events, both intracellular and S1PR-mediated. Regulation of S1P homeostasis is therefore of critical importance to numerous downstream signaling events.

S1P is produced by the action of sphingosine kinases, SphK1 and SphK2, which differ in tissue distribution, subcellular localization, and substrate specificity (20, 24, 26). Cellular S1P levels are further regulated by the activities of S1P phosphatases (SPPs) and S1P lyase (SPL), which dephosphorylate S1P to sphingosine or hydrolyze S1P to phosphoethanolamine and hexadecanol, respectively. Two mammalian SPPs have been identified that are highly specific for sphingosine phosphates (30, 33). Both are thought to function in the intracellular regulation of S1P and ceramide concentrations, or the ‘sphingolipid rheostat’, and therefore play a critical role in the determination of cell fate (23, 24). SPL irreversibly degrades S1P and the products serve as precursors to phospholipid synthesis, thus interconnecting the sphingolipid and phospholipid metabolic pathways (24, 51). SPL activity contributes to the regulation of apoptosis, migration, and developmental patterning in both simple organisms and in mice (5).

Both SphK isozymes as well as SPP1 and SPP2 are highly expressed in the mature mouse kidney (20, 26, 30, 33). Several studies report that S1P signaling has a potent influence on the renal response to ischemia-reperfusion injury (4, 16, 18), mesangial cell function, and the progression of diabetic nephropathy (13, 14, 19, 37). The regulation
and function of S1P during kidney development, however, has not been previously
reported. The current studies demonstrate mRNA expression of S1P metabolic enzymes
and receptors during development of the mouse metanephric kidney. SphK and SPP
activities also were examined in developing mouse kidneys and the resulting effect on
tissue S1P level was determined. To assess the impact of S1P homeostasis on kidney
morphogenesis, kidneys were cultured with a variety of SphK inhibitors. Results
demonstrate that S1P homeostasis is dynamically regulated during renal development by
differential expression of S1P metabolic enzymes and that SphK activity is essential for
kidney branching morphogenesis. Results further indicate that S1P receptor expression is
also differentially regulated in the developing mouse kidney.
MATERIALS AND METHODS

Animals. CD-1 mice were obtained from Charles River Laboratories (Wilmington, MA). Embryos were collected from timed pregnant females at indicated gestational ages (vaginal plug at E0.5). Kidneys were removed and processed for RT-PCR, enzyme activity, or organ culture as described below. To separate the ureteric bud from the metanephric mesenchyme, E11.5 kidneys were treated with 0.5 mg/ml collagenase B (Roche, Mannheim, Germany) in DMEM with 10%FBS at 37°C for 15 min, then transferred to fresh media and dissected using 30g needles. Handling, care and euthanasia of mice conformed to institutional animal care guidelines.

Real-time RT-PCR. Total RNA was isolated using the Micro-to-Midi RNA Isolation kit (InVitrogen, Burlington, ON, Canada), treated with DNase (InVitrogen), and reverse transcribed using Taqman reagents (Applied Biosystems, Foster City, CA). Real-time quantitative PCR was performed on an ABI PRISM 7500 system using TaqMan Gene Expression Assay products (Applied Biosystems) for SphK1 (Assay ID: Mm00448841_g1), SphK2 (Mm00445021_m1), SPP1 (Mm80473016_m1), SPP2 (Mm01158866_m1), SPL (Mm00486079_m1), S1P2 (Mm02620208_s1), S1P3 (Mm02620181_s1), S1P4 (Mm00468695_s1), and S1P5 (Mm02620565_s1). Primers and probe for S1P1 were 5’-CTACTCCTTGGTCAGGACTCGA, 5’-CGCCGCCTGACCTTCCGC, and 5’(HEX)-CGACTGGCCTTGGAGATGTT (IDT Inc., Coralville, IA). Cycling conditions were 95°C for 10 min followed by 95°C for 30 s and 60°C for 30 s cycled 45x. Expression was normalized using primer-limited VIC-labeled endogenous control primer-probe sets for β-actin or 18S rRNA (Applied
Biosystems). Relative expression was determined by comparison of dT values relative to expression of endogenous controls using the $2^{-\Delta\Delta C_T}$ method (27).

**SphK activity.** SphK activity was determined by a modification of previously described methods (35, 48). Kidneys were homogenized and sonicated in SphK buffer (20 mM Tris-HCl, pH 7.4; 20% glycerol; 1 mM β-mercaptoethanol; 1 mM EDTA; 1 mM Na orthovanadate; 15 mM NaF; 1 mM PMSF; protease inhibitor cocktail; 0.5 mM deoxypyrimidine; 40 mM β-glycerophosphate), then frozen at -80°C. Tissue lysates (50-100 μg protein) were incubated at 37°C for 30 min with 50 μM [$^3$H]sphingosine (40-50 dpm/pmol, American Radiolabeled Chemicals, St. Louis, MO) prepared in suspension with SphK buffer containing fatty acid-free BSA (0.02 mg/ml final concentration), 1 mM ATP, and 10 mM MgCl$_2$. Reactions were terminated by addition of 3 volumes methanol, vortexed, and extracted by addition of 6 volumes chloroform and 3 volumes 25 mM trisodium EDTA (pH 9.0). Following centrifugation, [$^3$H]S1P localized to the upper aqueous phase and activity was determined by scintillation counting. Extractions performed at time 0 were subtracted as background. Rates were corrected for extraction loss based on extraction efficiencies (~72%) determined using sphingosine substrate prepared with trace [$^3$H]S1P (American Radiolabeled Chemicals) instead of [$^3$H]sphingosine. Specific activity of the [$^3$H]sphingosine substrate was used to calculate SphK activity, which is expressed as pmol S1P formed/min/mg protein. Results were confirmed by comparison with SphK activity measured in adult kidney homogenates by the [$^{32}$P]ATP method (35).

**SPP activity.** SPP activity was determined by a modification of previously described methods (25). Kidneys were homogenized in SPP buffer containing 50 mM KPO$_4$ (pH
7.2), 0.02% Nonidet P-40, and 2 mM semicarbazide. Lysates were centrifuged at 100,000x g for 1 h to separate cytosolic and total membrane fractions. Membrane pellets were resuspended in SPP buffer. 10-50 µg membrane protein was incubated with 10 µM [³H]dihydro-S1P (0.5 µCi/ml; American Radiolabeled Chemicals) prepared in SPP buffer containing 0.3% fatty acid-free BSA in 200 µl reaction volume at 37°C for 60 min. Reactions were terminated by addition of 200 µl 7 M NH₄OH followed by 1 ml chloroform:methanol (3:2). Following centrifugation, [³H]dihydrosphingosine partitioned to the organic phase and was counted by liquid scintillation. Extractions performed on [³H]dihydro-S1P substrate without kidney homogenate were subtracted as background. SPP activity was calculated using the specific activity of the [³H]dihydro-S1P and reported as pmol/min/mg protein.

**S1P quantification.** S1P concentration in embryonic and adult kidneys was assessed by S1P ELISA (Echelon Inc., Salt Lake City, UT). Kidney homogenates prepared in SphK buffer described above were applied to the ELISA plate at 30 µg protein/well. ELISA was performed according to manufacturer’s instructions. Results were confirmed by comparison to S1P concentration determined by LC MS/MS, performed by the laboratory of A. Merrill.

**Kidney organ culture.** Metanephric kidneys isolated at age E11.5 were cultured on polyester filter discs (13 mm, 0.4 µm pore size, Whatman, Florham Park, NJ) floating atop culture medium (DMEM/Ham’s F-12 supplemented with 1% FBS, 1% L-glutamine, 1 µM dexamethasone, and 1% penicillin/streptomycin) in 12-well culture plates at 37°C; 5% CO₂ for 3-6 days similar to previously reported methods (40). FBS concentration was reduced to 1% to minimize the possible influence of serum albumin on
S1P concentration. N,N-dimethylsphingosine (DMS) and D,L-threo-dihydrosphingosine (DHS) were obtained from Sigma (St. Louis, MO). F-12509A and B-5354c were a generous gift from Dr. Takafumi Kohama (Daiichi Sankyo, Tokyo, Japan). SphK inhibitors were added to the culture media in EtOH (0.1% final concentration). Exogenous S1P (Sigma) was prepared in suspension with 4 mg/ml BSA in PBS and added to culture media at 1:100 dilution (10 µM final). Vehicle controls were treated with EtOH ± BSA only.

**Immunofluorescence.** To determine ureteric bud tip number, whole mount immunofluorescence was performed on cultured kidneys fixed in methanol at -20°C for 10 min, blocked with 1% BSA, then incubated with mouse anti-pancytokeratin antibody (1:200, Sigma), washed in PBS with 0.1% Tween-20 (PBST), and stained with FITC-conjugated anti-mouse secondary antibody (1:500, Sigma). Ureteric bud tip numbers were counted by epifluorescent or laser-scanning confocal microscopy.

**Apoptosis studies.** Apoptosis was assessed by DNA fragmentation analysis similar to previously described methods (38). Kidneys were washed twice with PBS, pelleted, and resuspended in 100 µl lysis buffer (10 mM EDTA, 20 mM Tris, pH 7.5, 0.5% Triton X-100). Samples were incubated on ice for 30 min with occasional inversion and centrifuged at 13,000x g for 20 min at 4°C. Lysates were then incubated at 37°C for 1 h with 0.2 mg/ml DNase-free RNase followed by 0.2 mg/ml proteinase K at 50°C for 2 h. DNA was precipitated at -20°C with an equal volume of isopropanol and 0.05 volume 5 M NaCl. Following centrifugation at 13,000x g for 15 min at 4°C, the DNA pellet was washed twice with 75% ethanol and dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4). DNA fragmentation was analyzed by electrophoresis in a 1.5% agarose gel.
**Statistical analysis.** Data are reported as means ± SE. Comparisons between groups were evaluated by unpaired Student’s *t*-test. Statistical significance was identified as P < 0.05.
RESULTS

*Expression and activity of S1P metabolic enzymes.* Transcriptional expression of genes encoding S1P metabolic enzymes was examined by real-time RT-PCR from induction of kidney morphogenesis at E11.5 through maturation. Fig. 1 illustrates developmental changes in renal expression of sphingosine kinases, SphK1 and SphK2, and S1P catabolic enzymes (SPP1, SPP2, and SPL). Expression of both SphKs and SPPs increased as development progressed (Fig. 1A). Expression of SphKs increased approximately 4-fold between induction at E11 and maturity, with increased expression of SphK1 preceding SphK2 at postnatal day 4. SphK2 was expressed at greater levels than SphK1 in both embryonic and mature kidneys (Fig. 1B). Expression of S1P phosphatases increased to a greater extent than SphKs (Fig. 1A). SPP2 expression increased approximately 8-fold and SPP1 increased more than 50-fold between age E11.5 and adult. SPP2 expression exceeded SPP1 in embryonic kidneys, while SPP1 was the predominant phosphatase in mature kidneys (Fig. 1B). SPL expression was relatively constant as development progressed, with a 2-fold increase observed at postnatal day 4. Importantly, the relative expression of kinases to catabolic enzymes changed as the kidney matured (Fig. 1B). In embryonic kidneys, SphK2 was expressed at higher levels than either SPPs or SPL, while in mature kidneys SPP expression was equal to or greater than expression of SphKs. Distribution of S1P metabolic enzyme expression within the embryonic kidney was further examined by RT-PCR following microdissection of the ureteric bud and metanephric mesenchyme (Fig. 2). Both SphKs and SPL were more highly expressed in the metanephric mesenchyme, while SPP2 expression was greater in the ureteric bud. Gene expression in the ureteric bud and metanephric mesenchyme were
normalized to 18S rRNA because β-actin was found to be more highly expressed in metanephric mesenchyme. GDNF expression is illustrated as both a positive control and for reference.

We examined SphK and SPP activities as well as S1P concentrations in embryonic (E14.5) and adult kidneys to determine whether the observed increases in mRNA expression result in corresponding changes in enzyme activity and a shift of S1P homeostasis (Table 1). SphK activity was fairly robust in adult kidney extracts at 122 ± 23 pmol/min/mg cellular protein and similar to previously reported results (12, 36). SphK activity in E14.5 kidneys was approximately 5-fold lower than observed for adult tissue at 21 ± 6 pmol/min/mg protein, reflecting differences observed in mRNA expression. SPP activity also mirrored changes in expression and was minimally detectable in kidneys at age E14.5 (2 ± 2 pmol/min/mg protein), while kidneys from adult mice exhibited SPP activity equal to or greater than SphK activity. S1P concentration assessed in embryonic and adult kidneys reflected the differential activities of S1P metabolic enzymes. S1P was relatively abundant at 287.7 ± 28.5 pmol/mg cellular protein in kidneys isolated at age E14.5, when activity of SphKs exceeded SPPs, while S1P was not detectable in adult tissue.

*SphK inhibitors reduce ureteric bud tip number in cultured kidneys.* Branching of the ureteric bud was examined in kidneys isolated at gestational day E11.5 and cultured for 3 days with increasing concentrations of the SphK inhibitor, N,N-dimethylsphingosine (DMS), or vehicle only (Fig. 3). Cytokeratin immunostaining shows numerous, symmetrical branch points and ampullary tips in vehicle controls (Fig. 3A; left) and a dramatic reduction in tip number with increasing concentrations of DMS (Fig. 3A; right).
Tip number was reduced by approximately 50% and 70% in the presence of 20 μM and 50 μM DMS, respectively (Fig. 3B). 10 μM DMS did not have a significant effect on tip number at 3 days of treatment (not shown). After 6 d of treatment, however, reduced ureteric bud branching was readily apparent with lower concentrations of DMS (Fig. 3C). DNA fragmentation analysis demonstrated increased apoptosis in kidneys treated with 20 and 50 μM DMS (Fig. 4). Apoptosis appeared equally distributed between the ureteric bud and mesenchyme in DMS-treated kidneys following TUNEL staining (not shown). Examination of DMS-treated kidneys by TUNEL staining did not reveal any specificity of apoptosis localization to ureteric bud or mesenchyme tissue. Addition of 10 μM exogenous S1P reduced the amount of DNA laddering compared to DMS treatment alone (Fig. 4). Exogenous S1P did not affect ureteric bud tip number in kidneys treated with or without DMS (not shown).

The effect of two novel SphK inhibitors, F-12509A and B-5354c, were tested to exclude the possibility of nonspecific effects associated with DMS treatment. F-12509A and B-5354c are structurally distinct from sphingosine and act as competitive and noncompetitive inhibitors, respectively (21, 22). Ureteric bud tip number was dose-dependently reduced in kidneys cultured in the presence of either of these inhibitors (Table 2). D,L-threo-dihydrosphingosine (DHS), a selective inhibitor of SphK1, also dose-dependently inhibited ureteric bud branching (Fig. 5). In contrast to kidneys treated with DMS, addition of exogenous S1P increased the number of ureteric bud tips in kidneys treated with DHS. Kidneys treated with 20 μM DHS plus exogenous S1P exhibited tip numbers similar to untreated kidneys, and kidneys treated with 50 μM DHS plus S1P had a significantly greater number of tips compared to kidneys treated with
DHS alone. Exogenous S1P also prevented apoptosis in DHS-treated kidneys similar to results obtained with DMS (not shown).

**Differential S1PR expression during kidney development.** Expression of S1P receptors was examined by quantitative RT-PCR in embryonic and mature kidneys (Fig. 6A). S1PR expression in the kidney was dependent on gestational age. S1P1 expression increased throughout kidney development and was the most highly expressed of the examined receptors in the adult kidney. Expression of S1P2 was approximately equal to S1P1 in kidneys isolated at age E11.5 and decreased toward maturity. Therefore, embryonic kidneys expressed both S1P1 and S1P2 while adult tissue expressed predominantly S1P1. Low levels of expression were also observed for S1P3 and S1P4 during early stages of kidney morphogenesis and S1P4 expression increased in mature kidneys, while S1P3 was undetectable in either embryonic or mature kidneys. Comparison of S1PR expression in mechanically separated ureteric bud and metanephric mesenchyme revealed differential expression of S1PRs within the developing kidney (Fig. 6B). Expression of both S1P1 and S1P2 was greater in the metanephric mesenchyme than in the ureteric bud.
DISCUSSION

This study demonstrates a dramatic shift in S1P homeostasis during kidney morphogenesis regulated by differential expression and activity of S1P metabolic enzymes. Despite lower SphK levels during early stages of kidney development, a near complete absence of S1P catabolism permits retention of S1P produced by SphKs. Furthermore, both SphK isotypes were expressed at greater levels in the metanephric mesenchyme, while SPP2 was more highly expressed in the ureteric bud. These results suggest that S1P is produced in greater amounts in the mesenchyme while S1P is depleted in the ureteric bud, generating an S1P gradient inclined toward the mesenchyme which may contribute to signals promoting ureteric bud outgrowth and mesenchyme survival. Indeed, SphK activity was essential for branching morphogenesis as SphK inhibition prevented ureteric bud branching and induced apoptosis in cultured metanephric kidneys.

Branching morphogenesis was inhibited by four different SphK inhibitors including DMS, DHS, and the structurally distinct inhibitors, F-12509A and B-5354C (21, 22). Independent mechanisms of SphK inhibition by each of these compounds limits the possibility that reduced branching was due to non-specific effects associated with any individual inhibitor. Furthermore, reduced branching in DHS-treated kidneys may indicate a critical role for SphK1 given that SphK2 is not effectively inhibited by DHS (26). Exogenous S1P rescued kidneys from apoptosis induced by SphK inhibition but did not affect branching of kidneys cultured with or without DMS, suggesting that S1P may influence ureteric bud branching and apoptosis by independent mechanisms. Interestingly, exogenous S1P restored ureteric bud branching in DHS-treated kidneys.
SphK1 activity may therefore be compensated, at least partially, by an exogenous source of S1P. Cell culture studies suggest that SphK1 and SphK2 have distinct, sometimes opposing functions (29). In contrast, studies of SphK1 and SphK2 knockout mice suggest some degree of functional redundancy in the activity of the two isozymes as neither SphK1(-/-) nor SphK2(-/-) mice exhibit obvious phenotypic abnormalities, yet double knockout mice do not survive beyond age E13.5 due to massive defects in the development of vascular and neuronal systems (2, 31). SphK1 and SphK2 may therefore contribute both independent and overlapping functions during kidney branching morphogenesis.

Differential expression of both SphK1 and SphK2 during development of the whole mouse embryo has been previously reported (26), yet mechanisms of developmental regulation have not been proposed. Most SphK activators act posttranslationally by a complex array of mechanisms including subcellular localization, phosphorylation, phosphatidic acid, Ca$^{2+}$, and interaction with adaptor proteins (1, 44). SphK activity is stimulated in response to a variety of growth and survival factors, contributing to their mitogenic and anti-apoptotic effects as well as chemotactic migratory responses (1, 24, 28). GDNF, TGFβ and EGF elicit a biphasic activation of SphK in other cell systems, with a rapid initial increase in enzyme activity followed by a prolonged increase in transcriptional expression (8, 32, 50). Early stages of kidney development are critically dependent on GDNF signaling (47), and subsequent branching of the ureteric bud and induction of mesenchymal transformation are further influenced by a multitude of growth factors, including EGF and TGFβ (7, 42). Transcriptional regulation of SphK expression
and posttranslational activation of SphKs may therefore contribute to mechanisms by which these growth factors regulate branching morphogenesis.

SPP expression and activity increased to a much greater extent than SphKs as development progressed, resulting in rates of S1P dephosphorylation equal to or greater than S1P production and a net reduction of S1P content in mature kidneys. Studies of sphingolipid content in kidneys of neonatal and adult rats found highest concentrations of S1P during the neonatal period that decreased with age, while ceramide and sphingomyelin content increased with age (11). Although these studies examined S1P content postnatally, the results are consistent with our findings of increased SPP activity and lower S1P content in mature kidneys. Regulation of S1P concentration by differential expression of S1P phosphatases poses intriguing possibilities for the establishment of S1P concentration gradients. SPP activity has also been reported to influence S1P secretion and may therefore contribute to the regulation of S1PR signaling activity (17). Further studies examining localized SPP expression and development of knockout mice deficient of SPP activity will undoubtedly shed light on the function of these enzymes during development. Relatively more is known about the regulation of SPL and its role in development (5). SPL is a transcriptional target of PDGF and SPL knockout mice display phenotypes consistent with PDGF signaling defects including congenital abnormalities in vascular, skeletal, and renal development (41). In the kidney, development of glomerular structures appears to be the primary site of disruption in SPL knockout mice and increased SPL expression during later stages of kidney development is consistent with the timing of glomerular development.
S1P₁ and S1P₂ were expressed at approximately equal levels in the embryonic kidney and both were predominately expressed in the metanephric mesenchyme. S1PRs regulate Rho GTPases and downstream cytoskeletal dynamics. S1P₁ activates Rac and is associated with increased cell motility, while S1P₂ activates Rho and inhibits both Rac activity and cell motility (34). Differential expression of these two S1PRs can therefore result in opposite responses to exogenous S1P. Several recent studies also indicate that cross-talk between S1PRs and growth factor receptors can modulate growth factor-induced cellular responses, providing another level of interaction between these two signaling pathways (6, 10, 45, 46).

S1P has been established as a key lipid mediator of developmental processes in multiple organ systems by operating an elaborate network of signals that regulate cell fate and proliferation, cytoskeletal rearrangements, and interactions with other cells and extracellular matrices necessary for migration and morphogenesis. Differential expression of S1P metabolic enzymes during metanephric kidney development results in dynamic regulation of S1P homeostasis as development progresses. This is the first report illustrating the critical role of SphK activity and S1P metabolism during kidney morphogenesis and, to our knowledge, the first study reporting developmental regulation of SPP gene expression. Differential expression of S1P receptors within the developing kidney likely contributes further to differential S1P-induced cellular responses. Further studies will undoubtedly reveal mechanisms by which S1P signaling contributes to the branching morphogenic program and advance our understanding of mechanisms regulating kidney morphogenesis.
GRANTS

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REFERENCES


FIGURE LEGENDS

Fig. 1. Expression of S1P metabolic enzymes during kidney development. (A) By real-time RT-PCR, mRNA expression of sphingosine kinases (SphK1, SphK2), S1P phosphatases (SPP1 and SPP2), and S1P lyase (SPL) increased as development progressed in kidneys isolated at prenatal days E11, E14, E17, postnatal day 4 (P4), and adult. All genes are normalized to β-actin and illustrated relative to expression at age E11. Values are means ± SE, n = 6 for each group. * P < 0.05, ** P < 0.01, *** P < 0.001 compared with expression at E11. (B) Comparison of S1P metabolic enzyme expression in embryonic (E11) and adult mouse kidneys.

Fig. 2. Expression of S1P metabolic enzymes in the ureteric bud (UB) and metanephric mesenchyme (MM) at age E11.5. Real-time RT-PCR of mRNA from E11.5 kidneys that were mechanically separated to yield ureteric bud and metanephric mesenchyme. Gene expression is illustrated relative to expression of 18S rRNA. Values are means ± SE. *P < 0.005 compared with expression in ureteric bud. n = 3; each sample was pooled from 28-32 kidneys.

Fig. 3. Effect of DMS on branching of the ureteric bud in cultured metanephric kidneys. (A) Wholemount immunofluorescence of E11 mouse kidneys cultured for 3 days in the absence (left, vehicle) or presence of increasing concentrations of DMS (right). DMS significantly reduced ureteric bud tip number in kidneys cultured for (B) 3
days or (C) 6 days. Values are means ± SE. *P < 0.005 compared with vehicle control. B: n = 6 for each group. C: 0 DMS, n = 40; 1 uM DMS, n = 29; 5 uM DMS, n = 29.

**Fig. 4. Effect of exogenous S1P on apoptosis in kidneys cultured with DMS.** DNA fragmentation analysis was performed on kidneys cultured for 3 days with 20 or 50 μM DMS in the presence or absence of 10 μM exogenous S1P. Lane 1, Culture media only; Lane 2, Vehicle control (0.1% EtOH + 4 mg/ml BSA); Lane 3, 20 μM DMS + 10 μM S1P; Lane 4, 20 μM DMS; Lane 5, 50 μM DMS + 10 μM S1P; Lane 6, 50 μM DMS; Lane 7, DNA size marker (M).

**Fig. 5. Effect of exogenous S1P on ureteric branching in kidneys cultured with DHS.** E11 kidneys were cultured for 6 days in the presence of 20 or 50 μM DHS ± 10 μM exogenous S1P. Tip numbers were determined by wholemount immunofluorescence. Values are means ± SE, n = 8 for each group. *P < 0.01 compared with vehicle control; #P < 0.05 compared with DHS alone.

**Fig. 6. Expression of S1P receptors (S1P1-5) (A) in embryonic (E11.5) and adult kidneys or (B) in separated ureteric bud (UB) and metanephric mesenchyme (MM) at age E11.5.** Gene expression was assessed by qRT-PCR and normalized relative to expression of β-actin (A) or 18S rRNA (B). Values are means ± SE. *P < 0.005 compared with expression at age E11.5 (A; n = 4) or expression in ureteric bud (B; n = 3, samples were pooled from 28-32 kidneys).
Table 1. Effect of SphK and SPP activities on S1P content in embryonic and adult kidneys.

<table>
<thead>
<tr>
<th>Age</th>
<th>SphK (pmol/min/mg protein)</th>
<th>SPP (pmol/min/mg protein)</th>
<th>S1P Content (pmol/mg protein)</th>
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<tbody>
<tr>
<td>E14.5</td>
<td>21.3 ± 6.1</td>
<td>2.0 ± 2.1</td>
<td>287.7 ± 28.5</td>
</tr>
<tr>
<td>Adult</td>
<td>122.1 ± 23.1*</td>
<td>158.1 ± 17.1*</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4 for each group. * P < 0.01 compared with E14.5 kidneys. ND: Not detectable.
Table 2. *Ureteric bud tip numbers in kidneys cultured for 6 days with SphK inhibitors, F-12509A or B-5354C*

<table>
<thead>
<tr>
<th>SphK Inhibitor</th>
<th>Tip number</th>
</tr>
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<tbody>
<tr>
<td>Vehicle</td>
<td>30.5 ± 2.7 (17)</td>
</tr>
<tr>
<td>F-12509A</td>
<td></td>
</tr>
<tr>
<td>10 µM</td>
<td>18.7 ± 3.0* (6)</td>
</tr>
<tr>
<td>25 µM</td>
<td>13.5 ± 1.9** (20)</td>
</tr>
<tr>
<td>50 µM</td>
<td>10.0 ± 2.3** (12)</td>
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<tr>
<td>B-5354C</td>
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<tr>
<td>5 µM</td>
<td>20.4 ± 1.7 (5)</td>
</tr>
<tr>
<td>10 µM</td>
<td>17.5 ± 4.2* (10)</td>
</tr>
<tr>
<td>20 µM</td>
<td>9.5 ± 1.7** (17)</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05, **P < 0.00001 compared with vehicle control. Numbers of kidneys for each treatment are indicated in parentheses.
AB Vehicle   DMS

DMS (PM) 015

Tip Number 0 10 20 30 40 50

DMS (PM) 02 0 5 0

Tip Number 0 5 10 15 20

*C

C 10 µM 20 µM 50 µM

A: Vehicle and DMS images at 10 µM, 20 µM, and 50 µM.

B: Graph showing tip number at 0, 20, and 50 µM DMS.

C: Graph showing tip number at 0, 1, and 5 µM DMS.
DHS (μM) Control 20 50

Tip Number

- S1P + S1P

DHS (μM) Control 20 50

Tip Number

- S1P + S1P