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INVERSE CORRELATION BETWEEN PLASMA SPHINGOSINE-1-PHOSPHATE AND CERAMIDE CONCENTRATIONS IN SEPTIC PATIENTS AND THEIR UTILITY IN PREDICTING MORTALITY

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ABSTRACT—**Introduction:** The aim of this study was to investigate the correlation between plasma sphingosine-1-phosphate (S1P) and ceramide concentrations in sepsis, and the possible mechanisms for altered expression. **Methods:** Plasma S1P and ceramide concentrations were measured by HPLC-ESI-MS/MS. HLA-DR (human leukocyte antigen-DR) expression on peripheral blood mononuclear cells was examined by flow cytometry. Platelet sphingosine kinases 1/2 (SphK1/2) mRNA expression, protein content, and enzyme activities were determined by qRT-PCR, western blot, and commercial enzyme assay kits, respectively. **Results:** Compared with healthy and ICU controls, septic patients had significantly decreased plasma S1P but increased ceramide concentrations ($P < 0.05$). S1P concentration was negatively associated with the ceramide concentration in the septic patients ($r = -0.36$, $P < 0.05$). Linear regression analysis found that plasma S1P and ceramide were linked not only to sequential (sepsis-related) organ failure assessment (SOFA) score but also the HLA-DR expression on circulating monocytes. An receiver operating characteristic analysis, including S1P, ceramide, SOFA score and HLA-DR, showed integrated analysis of S1P and ceramide as the better powerful predictors of septic lethality with area under the curve value of 0.95. More importantly, we found the platelet SphKs activities and the expression levels of SphK1 were significantly decreased in septic patients ($P < 0.05$). Linear regression analysis revealed platelet SphKs activity was positively associated with the plasma S1P concentration of the septic patients ($r = -0.41$, $P = 0.02$). **Conclusions:** Integrated analysis of plasma S1P and ceramide predict septic mortality with high accuracy. The decreased platelet SphK1 expression and subsequent reduced SphKs activity might be responsible for the decreased plasma S1P levels during sepsis.

KEYWORDS—Ceramide, platelet, sepsis, sphingosine kinases 1/2, sphingosine-1-phosphate

INTRODUCTION

Sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection (1). The current criterion for sepsis (Sepsis-3) has been suggested the increase of sequential organ failure assessment (SOFA) ≥ 2 points from baseline (1). In sepsis, the immune response that is initiated by an invading pathogen fails to return to homeostasis, thus culminating in uncontrolled inflammation and immune suppression, followed by organ dysfunction (2). So far, anti-sepsis treatment modalities are rather limited, and the mortality rate of these patients is approximately 25% to 30%, and even increases from 40% to 50% when shock is present (3, 4). Sepsis remains one of the most pressing public health burdens worldwide.

A main factor affecting sepsis-related mortality is the diagnostic delay that hinders proper treatment (5–7). Recognizing sepsis early improves outcomes of patients (8). Hallmarks for sepsis severity include uncontrolled inflammation and/or immune suppression, endothelial barrier disruption, and organ dysfunction, all of which are potentially regulated by sphingolipid mediators (9, 10). S1P and ceramide are 2 major sphigolipids, which contribute to membrane structure and signaling events that mediate response to stress such as sepsis (11, 12). Ceramide, generated by membrane sphingomyelin (SM) hydrolysis by sphingomyelinases (SMase),

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can act as a bioactive second messenger, or can be metabolized into sphingosine by ceramidases. Sphingosine is phosphorylated by sphingosine kinases 1 and 2 (SphK1/2) to generate S1P (13). There is a selective enrichment of SM in platelet cytoskeletons (13). SM is located in substantial amounts in the outer layer of platelet plasma membranes and is thought to be an important source of plasma S1P and ceramide (14). Ceramide and S1P exhibit opposite biological properties, ceramide promotes cell cycle arrest and apoptosis, whereas S1P exerts a pro-survival and mitogenic activity (13, 15). The inverse behaviors of both markers led us to compare the predictive value of S1P and ceramide analysis in sepsis-related mortality. In this study, we sought to determine whether serum S1P and ceramide levels are altered in sepsis based on the sepsis-3 definition; the predictive role of S1P and ceramide in the outcome of sepsis; and the possible reasons for the altered plasma-S1P and ceramide levels during sepsis.

METHODS

Study participants

Patients who met the clinical criteria for sepsis-3 were screened for eligibility within the first 24 h after they were admitted to the intensive care unit (ICU) of Zhejiang University Hospitals between September 2016 and May 2017 (16). A total of 33 septic patients were enrolled into the study. Patients were included if they had known or suspected infection plus an increase in the SOFA (sepsis-related) score of 2 points or more for organ dysfunction. Exclusion criteria were pregnancy or breast feeding, age younger than 18 yrs or older than 80 yrs, human immunodeficiency virus infection, treatment with corticosteroids or chemotherapy within 4 weeks, and inability to provide informed consent (17). Nineteen non-septic patients but in critical conditions with systemic inflammatory response syndrome (SIRS) were recruited as controls, such as trauma and severe acute pancreatitis. The clinical data, including SOFA score, cause of sepsis, microbial culture result, length of ICU stay, and mortality during the 28-day study period, were recorded (Table 1). Thirty-one healthy donors with no medical problems in the medical examination center of the affiliated hospitals of Zhejiang University were also included as controls. The study protocol was approved by the local institutional review board, and informed consent was obtained from all study participants or their surrogates.

S1P and ceramide measurement

S1P (C-18) and ceramide (C-16) in plasma isolated from study participants were quantified with high performance liquid chromatography-electrospray ionization tandem mass spectrometry/mass spectrometry as described before (17). Calibration curves for S1P measurement was prepared using 6 different concentrations ranging from 0 ng/mL to 10,000 ng/mL and for ceramide was prepared using 6 different concentrations ranging from 0 ng/mL to 500 ng/mL. FTY720 was used as an internal reference for each sample. The samples, including 25 μL of serum, 25 μL of internal standard, 25 μL of acetonitrile, and 25 μL of methanol, were mixed for 30 s on a rotator at room temperature and centrifuged at 18,400 g for 5 min. The supernatant was transferred to an autosampler HPLC vial, 8 μL of which was then injected into the HPLC system for analysis (Agilent Technologies, Santa Clara, CA). Quantification of the S1P and ceramide peaks was calculated from the standard calibration curve.

Peripheral blood mononuclear cells isolation

Peripheral blood was collected at day 1 of ICU admission. Monocytes were prepared as previously described (16). In brief, the blood was diluted with phosphate buffered saline (PBS). The diluted blood was then added gently to the top of the Ficoll solution (Sigma-Aldrich, St. Louis, MO; ID: F5415) and then centrifuged at 698 g for 20 min. The middle layer containing the mononuclear cells was then aspirated and washed twice with PBS. The cell pellets were resuspended in 1 mL Roswell Park Memorial Institute 1640 medium (Thermo Fisher Scientific, Rockford, IL) containing 10% fetal bovine serum (Moregate BioTech, Bulimba, Australia), and cultured in 12-well plates for 0.5 h in a humidified atmosphere at 37°C in 5% CO_2 and 95% air. The adherent monocytes were washed three times with PBS, and then were collected for flow cytometry analysis.

Peripheral blood platelet isolation

Platelets were prepared as previously described (18). Briefly, whole blood was collected into acid-citrate-dextrose (ACD) with 5 mM ethylene diamine tetraacetic acid (ACD: blood = 1:9, v/v). Platelet-rich plasma was collected by differential centrifugation at 160 g for 15 min. Platelets were isolated by centrifugation at 650 g for 15 min and then washed five times in PBS.

Flow cytometry analysis

In the case of blood samples, red blood cells were lysed with fluorescence activating cell sorter lysing solution. White blood cells were then washed and incubated with Fc block prior to staining with antibodies. The fluorescence-conjugated antibodies against CD14 (PerCP-Cy5.5) and human leukocyte antigen-DR (HLA-DR) (PE), both from eBioscience (eBioscience, San Diego, CA), were used for fluorescence activating cell sorter analysis. Cells were incubated with these antibodies for 20 min at 4°C in the dark, and then cell were washed and resuspended in 300 μL of PBS before applied to the BD LSR2 flow cytometer (BD Biosciences, San Jose, CA). The raw data were analyzed with the FlowJo software (FlowJo software Inc., Ashland, Ore).

Quantitative reverse transcription-polymerase chain reaction

Total RNA extraction and the subsequent first strand cDNA synthesis were prepared as previously described (17). Expression of SphK1 and SphK2 mRNA were assessed using relative quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (17). PCR reaction was performed in triplicate using

TABLE 1. **Patients characteristics**

Characteristics	Heath (n = 31)	ICU controls (n = 19)	Sepsis (n = 33)	*P* value
Age (yr)	52.71 ± 13.82	48.47 ± 15.34	57.45 ± 14.12	0.09
Sex, male (%)	21 (67.74%)	12 (63.16%)	27 (81.82%)	0.27
APACHE II score	NA	9.74 ± 4.48	12.24 ± 6.07	0.19
SOFA score	NA	5.16 ± 4.14	7.88 ± 4.14	0.027
Sepsis because of	NA	NA		
Peritonitis			21 (63.64%)	
Pneumonia			6 (18.18%)	
Urinary tract infections			2 (6.06%)	
Others			4 (12.12%)	
Microbe species	NA	NA	14 (42.42%)	
Escherichia coli			6 (18.18%)	
Staphylococcus aureus			2 (6.06%)	
Klebsiella pneumoniae			2 (6.06%)	
Pseudomonas aeruginosa			2 (6.06%)	
Candida albicans			2 (6.06%)	
Length of ICU study		1.89 ± 1.20	3.51 ± 3.10	0.034
28-day mortality		0 (0)	6 (18.18%)	0.075

Data are expressed as the mean ± SD or number (%) where applicable.
APACHE II, Acute Physiology and Chronic Health Evaluation II; ICU, intensive care unit; NA, not applicable; SOFA, sequential organ failure assessment.

Table 2. **Primers for polymerase chain reaction**

Gene	Sequence (from 5′ to 3′)
Human SphK1	Forward primer-CTGGCAGCTTCCTTGAACCAT
	Reverse primer-TGTGCAGAGACAGCAGGTTCA
Human SphK2	Forward primer-CGGTTGCTTCTATTGGTCAA
	Reverse primer-CGTTCTGTCTGGATGAGGTT
Human β-actin	Forward primer-AGAAAATCTGGCACCACACC
	Reverse primer-AGAGGCGTACAGGGATAGCA

ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). β-actin was used as an internal control. Relative expression level of SphK1 and SphK2 were determined using the comparative Ct method and referred to the expression level of the corresponding SphK1 in healthy controls which was set to 1. Primer sequences were described in Table 2.

Western blot

Samples were lysed in RIPA buffer (Santa Cruz Biotechnology, Santa Cruz, CA) and 15 μg of total lysate was resolved with 12% SDS-PAGE. Proteins were then transferred to PDVF membranes (Millipore, Billerica, MA). Membranes was blocked with 5% fat-free milk/PBS and blotted with antibodies against SphK1, Sphk2, or β-actin (Cell Signaling, Boston, MA). Membranes were subsequently washed and incubated in HRP-conjugated second antibodies (Millipore, Billerica, MA), washed, and detected with enhanced chemical luminescence (Biological Industries, Kibbutz Beit-Haemek, Israel) and exposed to X-ray films. The resulting protein bands were analyzed with Image J software.

SphK activity assay

SphKs activity in the platelets was measured using Echelon's Sphingosine Kinase Activity Assay kit (Echelon Biosciences, Salt Lake City, UT). In brief, cells were resuspended in ice-cold reaction buffer, and lysed with sonication and freeze/thaw cycles. Then the lysed cells were centrifuged at 10,000 *g* for 15 min. After determining the protein concentration using a bicinchoninic acid protein assay kit (Life Technologies, Grand Island, NY), equal amounts of protein was transferred to a 96-well white plate. The assay reaction was prepared by addition of sphingosine (substrate) in reaction buffer and initiated by the addition of ATP. The reaction was then stopped by adding the ATP detector after incubation of 2 h at room temperature. The emitted luminescence was detected by a luminometer (Molecular Devices, Sunnyvale, CA), and a buffer blank was subtracted from each reading. The luminescent signal is inversely correlated with the kinase activity.

Neutral sphingomyelinase activity assay

Neutral sphingomyelinase (N-SMase) activity in the platelets was measured using Echelon's Sphingosine Kinase Activity Assay kit (Echelon Biosciences, Salt Lake City, UT). It is an enzyme-coupled assay that measures sphingomyelinase activity in biological samples through the downstream production of choline. Briefly, 100 μL of reaction mixture was added to each well of microtiter plate, then 100 μL of samples or sphingomyelinase standard was added to microtiter plate. After incubating the microtiter plate at 37°C for 4 h with shaking, the absorbance was detected at 595 nm using a microplate reader (Molecular Devices, Sunnyvale, CA). The absorbance signal is positively correlated with the kinase activity.

Statistical analysis

The data in figures are presented as mean ± SD unless stated otherwise. A two-tailed unpaired *t*-test was used to compare differences in continuous variables between two groups. Multi-group comparisons were assessed using analysis of variance (ANOVA) followed by Bonferroni *post hoc* tests. Differences in categorical data between two groups were compared using Fisher exact test or Chi-squared test. Correlations were analyzed by using linear regression. Receiver operating characteristic curves were generated to determine sensitivity versus 1-specificity, and areas under the curve (AUC) were calculated to examine the sensitivity. All statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, Ill) or Prism 6.0 (GraphPad software Inc., La Jolla, Calif) software, and $P < 0.05$ was considered statistically significant.

RESULTS

Concentrations of plasma S1P and ceramide change oppositely in patients with sepsis

Thirty-three septic patients, 19 ICU non-septic controls (including 8 traumas and 11 SAP) and 31 healthy controls with similar demographic characteristics were enrolled into the study. The plasma levels of S1P and ceramide were measured by high performance liquid chromatography-electrospray ionization tandem mass spectrometry/mass spectrometry after de-proteinization of serum with acetonitrile. Compared to those in healthy and ICU controls (1011.35 ± 146.34 ng/mL in health, 954.48 ± 174.86 ng/mL in ICU controls), the plasma S1P levels were significantly decreased in septic patients (706.69 ± 139.66 ng/mL, $P < 0.05$) (Fig. 1A). In contrast, the septic patients had significantly increased ceramide levels (47.17 ± 20.63 ng/mL, $P < 0.001$) when compared to the controls (20.65 ± 11.96 ng/mL in health, 26.61 ± 16.7 ng/mL in ICU controls) (Fig. 1B). Moreover, S1P levels were negatively associated with the ceramide levels in the septic patients (regression coefficient (r) = −0.36, $P < 0.05$, Fig. 1C).

Plasma S1P and ceramide concentrations are linked to disease severity and immune status

SOFA score is a particularly useful predictor of disease severity in critically ill patients (19). Linear regression analysis revealed a strong inverse correlation between SOFA scores and plasma S1P levels with a regression coefficient (r) of −0.50 ($P < 0.01$; Fig. 2A), and a strong positive correlation between

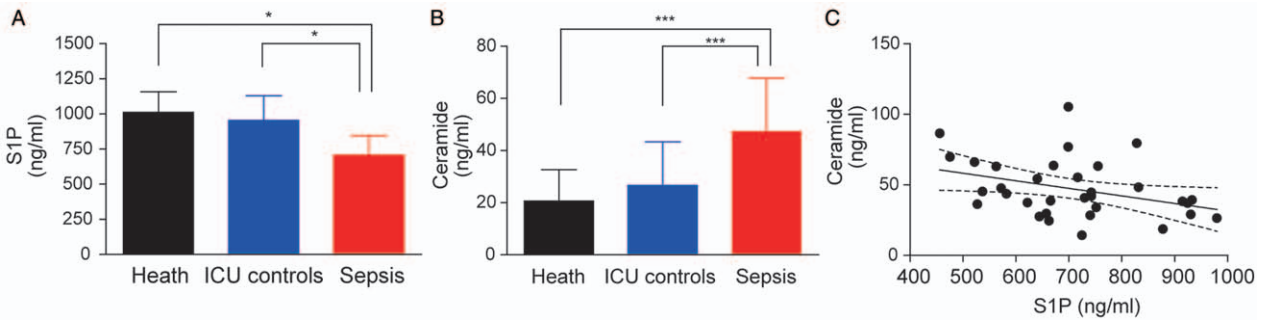

Fig. 1. **S1P and ceramide concentrations in plasma from the studied patients. S1P (A) and ceramide (B) concentrations in plasma from different groups. n = 31 for healthy controls, n = 19 for ICU critical control, and n = 33 for sepsis patients.** The data are shown as the mean ± SD and were analyzed by one-way ANOVA with Bonferroni corrections for multiple comparisons. (C) Correlation of S1P levels with ceramide levels in the septic patients ($r = -0.36$, $P < 0.05$). The data were analyzed by using linear regression analysis with 95% confidence line. $^{*}P < 0.05$, $^{***}P < 0.001$. ICU, intensive care unit; S1P, sphingosine-1-phosphate.

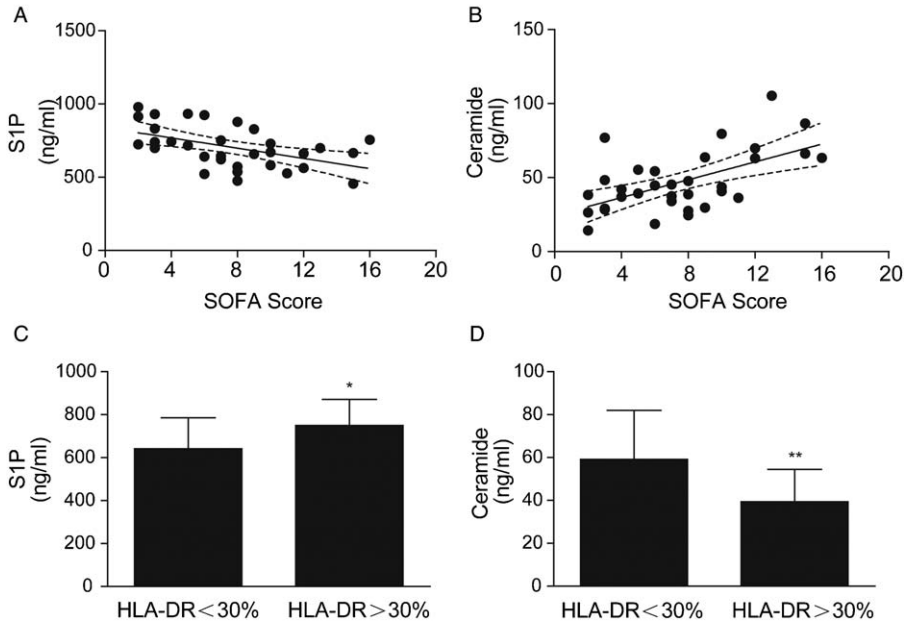

FIG. 2. **Association of S1P and ceramide levels in plasma from septic patients with the organ functions and immune status. Correlation of S1P (A) and ceramide levels (B) with SOFA scores in the septic patients (S1P: $r = -0.5$, $P < 0.01$; ceramide: $r = -0.58$, $P < 0.001$).** The data were analyzed by using linear regression analysis with 95% confidence line. (C and D) S1P and ceramide levels in septic patients which were divided into two groups according to the expression level of HLA-DR (<30% and >30%) in monocytes. n = 13 in the HLA-DR < 30% group, n = 20 in the HLA-DR>30% group. The data are shown as the mean ± SD and were analyzed by Student's *t* test. $^{*}P < 0.05$, $^{**}P < 0.01$. HLA-DR, human leukocyte antigen-DR; S1P, sphingosine-1-phosphate; SOFA, sequential organ failure assessment score

SOFA scores and plasma-ceramide levels with a regression coefficient (r) of 0.58 ($P < 0.001$; Fig. 2B).

Previous studies have documented that some septic patients have severe immune alterations and are at high risk of death or nosocomial infection. Down-regulation of HLA-DR on circulating monocytes is one of the surrogate markers for immune alteration (20, 21). To demonstrate that levels of plasma S1P and ceramide were linked not only to disease severity but also to immune status, septic patients were classified into two groups according to the expression of HLA-DR on circulating monocytes. In those patients with decreased expression of HLA-DR (<30%), S1P expression was significantly lower than that in patients with higher HLA-DR expression (>30%), whereas ceramide showed the opposite trend (Fig. 2C and D).

Integrated analysis of plasma S1P and ceramide predict septic mortality with high accuracy

To compare the potential of various clinical parameters with S1P and ceramide as to indicate mortality within patients with sepsis, an ROC analysis with S1P, ceramide, ceramide/S1P ratio, SOFA score, and HLA-DR was performed. Among all parameters tested, plasma S1P and the ceramide/S1P ratio emerged as the better powerful indicators of lethality, with AUC values of 0.94 for S1P and 0.95 for ceramide/S1P ratio (Fig. 3). The ROC curves for ceramide, SOFA, and HLA-DR were similar, with AUC values between 0.82 and 0.85.

Platelets release S1P and contribute to plasma S1P levels during sepsis

It has been known that platelets, which are primarily involved in hemostasis, are decreased in septic individuals, and the more profound the thrombocytopenia, the more severe the sepsis and the greater the mortality (22, 23). In our current study, the platelet count in the septic patients and ICU controls are comparable, both of which are lower than that in the health (Fig. 4AA). So, thrombocytopenia may not distinguish sepsis from ICU controls.

It also should be noted that, during coagulation, S1P released from platelets contributed to total plasma-S1P levels (24, 25).

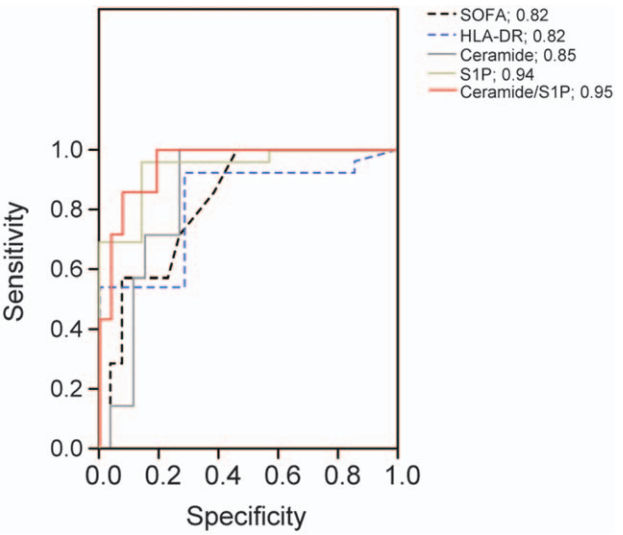

FIG. 3. **ROC curves for the prediction of death in septic patients.** AUC, area under the curve; HLA-DR, human leukocyte antigen-DR; ROC, receiver operating characteristic; S1P, sphingosine-1-phosphate; SOFA, sequential organ failure assessment score.

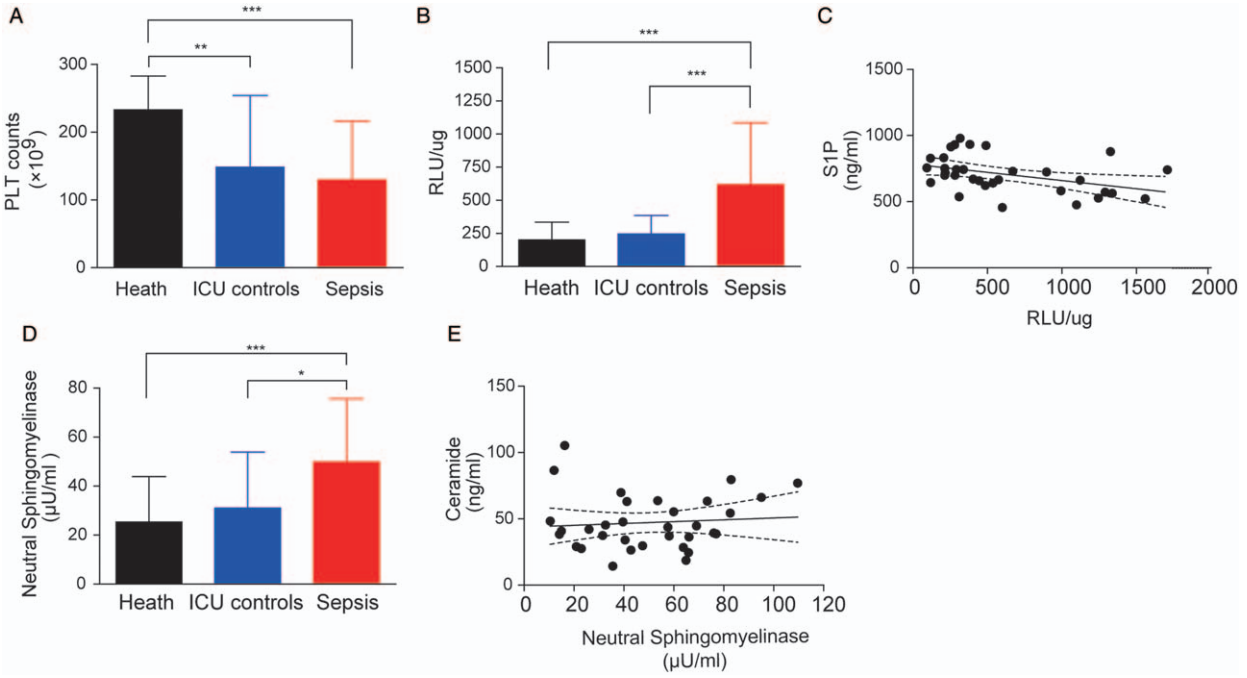

FIG. 4. **Analysis of platelet counts, platelet SphKs, and neutral sphingomyelinase activities in the study participants.** (A) Analysis of platelet counts in health and ICU patients. n = 31 for healthy controls, n = 19 for ICU critical controls, and n = 33 for sepsis patients. The data are shown as the mean ± SD and were analyzed by one-way ANOVA with Bonferroni corrections for multiple comparisons. (B) SphKs activities in platelets isolated from the studied patients were analyzed by an ATP depletion assay. The luminescent signal is inversely correlated with the kinase activity. The data are shown as the mean ± SD and were analyzed by one-way ANOVA with Bonferroni corrections for multiple comparisons. (C) Correlation of S1P with SphKs activity in the septic patients ($r = -0.41$, $P < 0.05$). The data were analyzed by using linear regression analysis with 95% confidence line. (D) N-SMase activities in platelets from the studied patients. The data are shown as the mean ± SD and were analyzed by one-way ANOVA with Bonferroni corrections for multiple comparisons. (E) Correlation of ceramide with N-SMase activity in the septic patients ($P = 0.63$). The data were analyzed by using linear regression analysis with 95% confidence line. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. N-SMase, neutral sphingomyelinase; RLU, relative light unit; SphKs, sphingosine kinases.

Rapid and profound depletion of plasma S1P during systemic anaphylaxis rendered platelet-derived S1P essential for survival (26). To assess whether platelet is responsible for the alterations of plasma S1P and ceramdie concentrations in septic patients, we evaluated the platelet sphingosine kinases (SphKs) and N-SMase activities in the studied patients. As expected, the SphKs activity in the 33 septic patients was significantly lower than in the health and ICU controls (Fig. 4B). The SphKs activities detected from the health and ICU controls were comparable (Fig. 4B). Linear regression analysis revealed SphKs activities were positively associated with the plasma S1P concentration of the septic patients ($r = -0.41$, $P = 0.02$, Fig. 4C). Contrarily, compared with the health and ICU controls, the N-SMase activity was significantly increased in septic patients (Fig. 4D). However, Linear regression analysis revealed the increased N-SMase activity was not the major reason for the increased plasma ceramide in septic patients ($P = 0.63$, Fig. 4E).

Reduced platelet SphK1 expression leads to the decreased SphKs activities in septic patients

To explore the reason of the decreased platelet SphKs activities in septic patients, we further evaluated the platelet SphK1/2 mRNA and protein levels in the studied patients. The mRNA level of SphK1 was ~15-fold higher than SphK2 in platelets isolated from the health controls (Fig. 5A). Compared with the health and ICU controls, SphK1 mRNA was significantly lower from septic patients (Fig. 5B). Platelet SphK1 protein expression was also significantly decreased in patients with sepsis (Fig. 5D). However, the SphK2 mRNA and protein levels were comparable in those study participants (Fig. 5, C and D).

DISCUSSION

The main findings of our current study are that patients with sepsis-3 have decreased plasma S1P but increased plasma-ceramide concentrations. This phenomenon was also confirmed in the patients with sepsis-1 (Supplemental Table 1 and Supplemental Figure 1, http://links.lww.com/SHK/A793). Integrated analysis of plasma S1P and ceramide predict septic mortality with high accuracy. Furthermore, reduced plasma S1P levels may result from lower platelet SphK1 expression and SphKs activity during sepsis.

Recognizing sepsis early improves patient outcomes (8). The commonly used plasma biomarkers of inflammation or infection, such as procalcitonin and C-reactive protein, identify several conditions characterizing sepsis and thus help to confirm the diagnosis (27). However, these biomarkers have limited use in preventing sepsis-related multi-organ dysfunction syndrome. Ceramide and S1P have emerged as a new class of lipid biomodulators of various cell functions (10). Ceramide

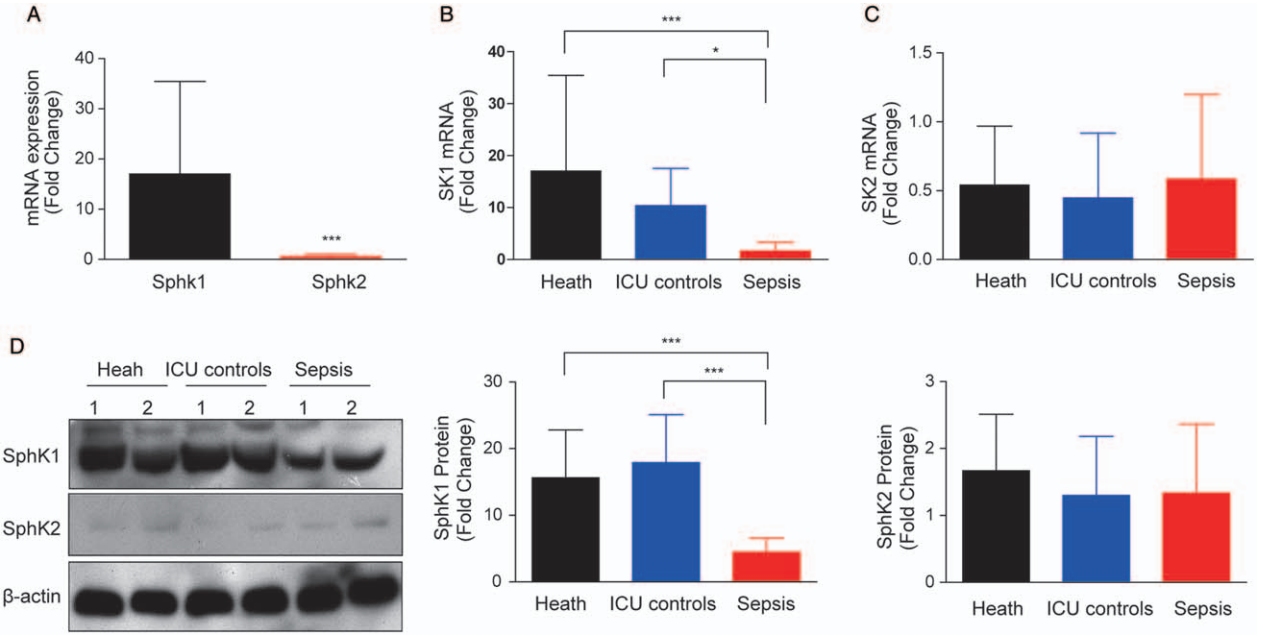

FIG. 5. **SphK1/2 expression levels in the studied patients.** (A) SphK1 and SphK2 mRNA levels in platelets isolated from the health controls. n = 31 for healthy controls. The data are shown as the mean ± SD and were analyzed by Student's *t* test. (B and C) SphK1 and SphK2 mRNA levels in platelets isolated from the studied patients. n = 31 for healthy controls, n = 19 for ICU controls, and n = 33 for septic patients. The data are shown as the mean ± SD and were analyzed by one-way ANOVA with Bonferroni corrections for multiple comparisons. (D) SphK1 and SphK2 protein levels in platelets isolated from the studied patients. n = 12 for healthy controls, n = 12 for ICU controls, and n = 18 for septic patients. The data are shown as the mean ± SD and were analyzed by one-way ANOVA with Bonferroni corrections for multiple comparisons. $^{*}P < 0.05$, $^{***}P < 0.001$. ICU, intensive care unit; SphK1/2, sphingosine kinase 1/2.

is shown to mediate many cellular events including growth arrest, differentiation, and apoptosis (11, 13). In contrast to ceramide, S1P has been implicated in mediating cell proliferation and antagonizing ceramide-mediated apoptosis (15). In patients with sepsis, we found plasma S1P was approximately 30% lower than in controls. In contrast, ceramide level had nearly 2-fold increase in septic patients. Linear regression analysis revealed a strong inverse correlation between SOFA scores and plasma S1P levels, and a strong positive correlation between SOFA scores and plasma-ceramide levels. These results suggested that the imbalance of ceramide/S1P ratio creates the SIRS and subsequent organ dysfunction during sepsis.

The degree of HLA-DR expression or the response of leukocytes to pathogenic stimuli reflects the patients' host response (28). The expression of HLA-DR in health is up to 90% (the rate of HLA-DR positive monocytes/total monocytes). Down-regulation of HLA-DR on the surface of circulating monocytes is generally accepted as a reliable marker for an immune dysfunction in septic patients. Importantly, lowered expression of ≤30% HLA-DR has been associated with the severity of sepsis, especially with fatal outcome and septic shock (28–30). Therefore, a cutoff of 30% HLA-DR expression was used to stratify the septic patients. In present study, in those with decreased expression of HLA-DR (<30%), S1P levels were significantly reduced than those in patients with higher HLA-DR expression (>30%). However, ceramide showed the opposite trend. The imbalance of ceramide/S1P ratio may induce the cycle arrest and apoptosis of immune cells, and then promotes the subsequent immune dysfunction.

Platelets are potent immune modulators and effectors (31). Platelets abundantly express SM at the cell surface, which is thought to be an important source of plasma S1P and ceramide (13, 14). The amount of S1P released from platelets is much higher than that from other cell types, as it possesses a highly active SphKs and do not have S1P lyase or S1P phosphatase activity (32, 33). During coagulation and systemic anaphylaxis, platelets release S1P and contribute to total circulating S1P levels and survival (25, 26). In our current study, we found SphK1 is the major SphKs in human platelets. Compared with the health and ICU controls, SphK1 expression and its activity are significantly decreased in platelets from septic patients. Linear regression analysis revealed SphKs activity in platelet was positively associated with the plasma S1P concentration of the septic patients. The SphK1 mRNA and protein responses were nicely correlated in platelets. Therefore, measures of genetically up-regulate the SphK1 mRNA content in platelet maybe a potential way to increase the protein translation and rescue the dysfunctional platelets during sepsis.

N-SMase is a key SMase for ceramide generation that is involved in regulating cellular stress responses and exosome-mediated intercellular communication (34). In our current study, compared with the health and ICU controls, the platelet N-SMase activity was significantly increased in septic patients. Tani *et al*. demonstrated that treatment with bacterial SMase results in sphingosine generation at the platelet extracellular surface (24). Schissel *et al*. also showed the secretory sphingomyelinase activity, which mediates the extracellular degradation of SM to ceramide during inflammation (35). So, the sSPMase-mediated ceramide production may be associated

with the development of sepsis. That may be the reason why linear regression analysis revealed the increased N-SMase activity in platelet was not associated with the increased plasma ceramide in septic patients. In summary, our study demonstrates that plasma S1P is negatively correlated with ceramide in septic patients and is more powerful than ceramide as a predictor of sepsis mortality. The events contributing to the decreased plasma S1P concentration, according to our findings, is depended on the dysfunctional platelets in SphK1 expression. This pilot study implies that pharmaceutical agents targeting platelets SphK1 may have beneficial value for septic patients.

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