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ORIGINAL ARTICLE

Role of Sterile 20/SPS1-related Proline/Alanine-rich Kinase in Mice with Endotoxic Shock

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Background: Na^+-K^+-$2Cl^-$ co-transporters (NKCCs) are involved in the regulation of permeability and tissue edema during sepsis. Inhibition of NKCC can reduce inflammation, edema formation, and bacterial burden. STE20/SPS1-realted proline/alanine-rich kinase (SPAK) is known to phosphorylate and activate NKCCs. However, there is no data regarding the role of SPAK in the pathological responses of sepsis. Therefore, the aim of this study was to examine the changes of systemic responses to endotoxemia in SPAK knockout mice. **Materials and Methods:** Wild-type and SPAK knockout mice were randomly given with vehicle (saline) or *Escherichia coli* lipopolysaccharide (LPS, 50 mg/kg) and monitored for 24 h. The alterations of hemodynamics, blood glucose, biochemical variables, plasma nitric oxide (NO) levels, blood flow, superoxide levels, and survival rate were analyzed during the experimental period. **Results:** In this study, LPS induced circulatory failure, hypoglycemia, multiple organ dysfunction, and mortality in wild-type mice. The NO levels of plasma were augmented and blood flow of the tongue, palm, sole, and abdomen were reduced in wild-type mice with endotoxic shock. However, there were no significant differences in these functional parameters and survival rate between wild-type and SPAK knockout mice with endotoxemia. **Conclusions:** These results demonstrate that inhibition of SPAK did not improve circulatory failure, hypoglycemia, multiple organ dysfunction, or mortality in mice that treated LPS. Thus, it seems that SPAK may not play an important role in endotoxic shock.

Key words: STE20/SPS1-realted proline/alanine-rich kinase, endotoxic shock, multiple organ dysfunction syndrome, nitric oxide, superoxide

INTRODUCTION

Sepsis is a high-burden healthcare disease caused by microbial invasion and immune dysregulation.[1] The major component of Gram-negative bacteria, lipopolysaccharide (LPS), can lead to systemic inflammatory response through excessive production of cytokines, reactive oxygen species (ROS), and nitric oxide (NO).[2] Progressive circulatory failures would result in the development of cellular hypoxia and multiple organ dysfunction syndrome during sepsis. The loss of organ function is considered to be the promoter of high mortality in patients. Thus, identification of therapeutic approaches to improve the outcome of sepsis is important.

Na^+-K^+-$2Cl^-$ co-transporters (NKCCs) belong to the family of solute carriers involved in the transport of ions across membranes and maintenance of electrochemical gradient.[3,4] NKCC1 is expressed in many cells, including endothelial cells, and plays an important role in regulating permeability. Increased permeability contributes to the shift of circulating components and tissue edema in sepsis.[5] It has been demonstrated that NKCC1 of brain microvascular endothelial cells participates in edema formation during cerebral ischemia.[6,7] Inhibition of

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NKCC1 can attenuate the increase of vascular permeability and the course of inflammatory responses.[6,8-11] In addition, mice lacking the NKCC1 are protected from lung bacterial burden and the development of bacteremia.[12] Taken together; these findings indicate the importance of NKCC1 in the regulation of inflammation and sepsis.

STE20/SPS1-realted proline/alanine-rich kinase (SPAK) is an enzyme that can phosphorylate NKCCs on N-terminal conserved domain and increase their activities.[13] NKCC1 phosphorylation is decreased in the aorta of SPAK-deficient mice when compared with wild-type mice.[14] The contribution of the SPAK to ion transport and fluid secretion has been well established. Far less is known about the role of this enzyme in the pathological responses of sepsis. Thus, we compared the systemic responses to endotoxemia between wild-type mice and SPAK knockout mice to characterize the functional implication of SPAK in sepsis.

MATERIALS AND METHODS

Animal and experimental procedures

This study was with approval of the Institutional Animal Care and Use Committee of National Defense Medical Center (Taipei, ROC, Taiwan) (Permit Number: IACUC-13-213). SPAK knockout mice were the gifts from Dr. Sung-Sen Yang (Division of Nephrology, Department of Medicine, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan) as described previously.[14] All mice were bred and maintained under the 12 h light/dark cycle with free access to food and tap water. 10–20-week-old male mice were used in this study. Animals were randomly divided into 6 groups and given with vehicle (saline) or *Escherichia coli* (LPS, 50 mg/kg) intraperitoneally: $SPAK^{+/+}$ + vehicle, $SPAK^{+/-}$ + vehicle, $SPAK^{-/-}$ + vehicle, $SPAK^{+/+}$ + LPS, $SPAK^{+/-}$ + LPS, and $SPAK^{-/-}$ + LPS. The experiments were monitored for 24 h. During the experimental period, we observed the changes in hemodynamics (i.e., systolic blood pressure and heart rate). Blood samples were collected at 24 h to examine the alterations of blood glucose, cell injury index (i.e., lactate dehydrogenase [LDH]), hepatic function index (i.e., alanine aminotransferase [ALT]), renal function index (i.e., blood urea nitrogen [BUN] and creatinine [CRE]), and plasma NO levels. At 24 h after vehicle or LPS, blood flow of the tongue, palm, sole, and abdomen in mice were also measured. Then the mice were sacrificed by overdosed pentobarbital, and lungs, livers, kidneys, and aortas were exercised to analyze superoxide production. The survival rate was assessed during the experimental period.

Recording of blood pressure and heart rate

Systolic blood pressure and heart rate of mice were measured by tail-cuff method using the MK-2000A blood pressure monitor (Muromachi Kikai, Tokyo, Japan). At baseline (i.e., time 0) and 3, 9, 21, 24 h after vehicle or LPS, blood pressure and heart rate were monitored.

Measurement of blood glucose

Blood samples were collected at 24 h after vehicle or LPS. Ten microliters of blood were used to evaluate the glucose levels by the One Touch II blood glucose monitoring system (Lifescan, Milpitas, CA, USA).

Quantification of organ function

Blood samples were drawn at 24 h after vehicle or LPS and then centrifuged at 16,000 g for 2 min to obtain the serum for assessing biochemical variables. LDH, ALT, BUN, and CRE were analyzed by Fuji DRI-CHEM 3030 (Fuji Photo Film, Tokyo, Japan).

Recording of blood flow

The mice were anesthetized with sodium pentobarbital at 24 h after vehicle or LPS. Blood flow around the tongue, palm, sole, and abdomen area were recorded using laser speckle contrast imager (Moor Instruments, Devon, UK).

Determination of superoxide production in organs

Lung, liver, kidney and thoracic aorta were obtained from the mice at the end of experiments. They were incubated with Krebs-HEPES buffer and then transferred to 96-well microplates containing Krebs-HEPES buffer with 1.25 mM lucigenin. Luminescence counts were obtained by using a microplate luminometer (Hidex Microplate Luminometer, Finland). All organs were dried in the oven, and the levels of superoxide were expressed as count per second per milligram of organ dry weight.

Assessment of plasma nitric oxide levels

Plasma collected at 24 h after vehicle or LPS was deproteinized by incubating with 95% ethanol and subsequently centrifuged at 16,000 g for 6 min. The amounts of plasma NO were determined by an NO analyzer (Sievers 280 NOA; Sievers Inc., Boulder, CO, USA) after adding the reducing agent (0.8% VCl_3 in 1 N HCl) to the purge vessel. The concentration was analyzed from a curve created from the standard solutions of sodium nitrate (Sigma Chemical Co., St Louis, MO, USA).

Statistical analysis

All data are expressed as mean ± standard error of mean of n determinations, where n represents the number of mice studied. Statistical significance between groups was performed by one-way analysis of variance followed by a multiple

comparison test (student-Newman-Keuls test). P <0.05 was considered to be significant.

RESULTS

Hemodynamic parameters in wild-type or STE20/SPS1-realted proline/alanine-rich kinase knockout mice treated with lipopolysaccharide

The LPS caused a significant fall in systolic blood pressure from 3 to 24 h, and a significant decrease in heart rate from 9 to 24 h in wild-type and SPAK knockout mice [Figure 1a and b]. However, there were no significant differences in systolic blood pressure and heart rate among SPAK$^{+/+}$ + LPS, SPAK$^{+/-}$ + LPS, and SPAK$^{-/-}$ + LPS groups [Figure 1a and b].

Blood glucose and nitric oxide levels in wild-type or STE20/SPS1-realted proline/alanine-rich kinase knockout mice treated with lipopolysaccharide

The level of blood glucose was significantly decreased at 24 h after LPS administration in wild-type and SPAK knockout mice [Figure 2a]. There were no significant differences in blood glucose among SPAK$^{+/+}$ + LPS, SPAK$^{+/-}$ + LPS and SPAK$^{-/-}$ + LPS groups [Figure 2a]. In addition, LPS elicited a significant elevation in plasma NO level in wild-type and SPAK knockout mice [Figure 2b]. However, the level of NO was not different among SPAK$^{+/+}$ + LPS, SPAK$^{+/-}$ + LPS, and SPAK$^{-/-}$ + LPS groups [Figure 2b].

Organ function in wild-type or STE20/SPS1-realted proline/alanine-rich kinase knockout mice treated with lipopolysaccharide

At 24 h after LPS, the levels of LDH, ALT, BUN, and CRE were significantly increased in wild-type and SPAK knockout mice [Figure 3a-d]. However, all these functional indexes were not different among SPAK$^{+/+}$ + LPS, SPAK$^{+/-}$ + LPS, and SPAK$^{-/-}$ + LPS groups [Figure 3a-d].

Blood flow in wild-type or STE20/SPS1-realted proline/alanine-rich kinase knockout mice treated with lipopolysaccharide

Wild-type and SPAK knockout mice showed significant decreases in blood flow of the tongue, palm, sole, and abdomen at 24 h after LPS administration [Figure 4a-d]. However, the blood flow of these areas was not different

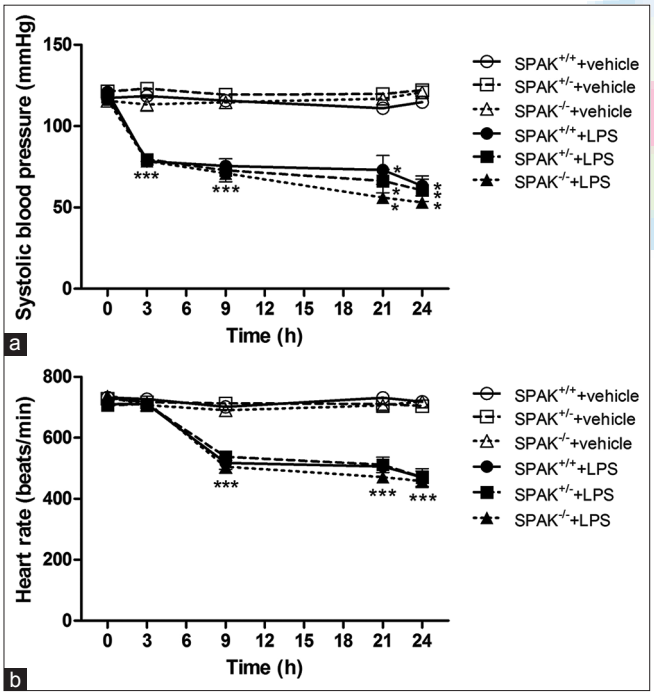

Figure 1: The changes of (a) systolic blood pressure and (b) heart rate during the experimental period in wild-type or SPAK knockout mice treated with lipopolysaccharide. Depicted are SPAK$^{+/+}$ mice that received saline (SPAK$^{+/+}$ + vehicle, n = 14) or lipopolysaccharide (SPAK$^{+/+}$ + lipopolysaccharide, n = 12), SPAK$^{+/-}$ mice that received saline (SPAK$^{+/-}$ + vehicle, n = 12) or lipopolysaccharide (SPAK$^{+/-}$ + lipopolysaccharide, n = 12), and SPAK$^{-/-}$ mice that received saline (SPAK$^{-/-}$ + vehicle, n = 8) or lipopolysaccharide (SPAK$^{-/-}$ + lipopolysaccharide, n = 10). Data are expressed as mean ± standard error of mean *P < 0.05, all versus SPAK$^{+/+}$ + vehicle mice; $^{\#}P$ < 0.05, without versus with SPAK in lipopolysaccharide mice. SPAK = STE20/SPS1-realted proline/alanine-rich kinase

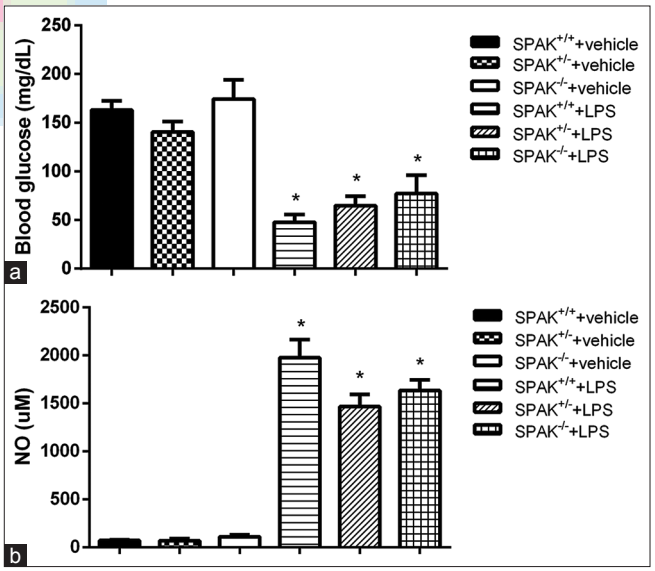

Figure 2: The changes of (a) blood glucose and (b) nitric oxide (NO) at the end of experiment (at 24 h) in wild-type or SPAK knockout mice treated with lipopolysaccharide. Depicted are SPAK$^{+/+}$ mice that received saline (SPAK$^{+/+}$ + vehicle, n = 14) or lipopolysaccharide (SPAK$^{+/+}$ + lipopolysaccharide, n = 12), SPAK$^{+/-}$ mice that received saline (SPAK$^{+/-}$ + vehicle, n = 12) or lipopolysaccharide (SPAK$^{+/-}$ + lipopolysaccharide, n = 12), and SPAK$^{-/-}$ mice that received saline (SPAK$^{-/-}$ + vehicle, n = 8) or lipopolysaccharide (SPAK$^{-/-}$ + lipopolysaccharide, n = 10). Data are expressed as mean ± standard error of mean *P < 0.05, all versus SPAK$^{+/+}$ + vehicle mice; $^{\#}P$ < 0.05, without versus with SPAK in lipopolysaccharide mice. SPAK = STE20/SPS1-realted proline/alanine-rich kinase

among SPAK$^{+/+}$ + LPS, SPAK$^{+/-}$ + LPS, and SPAK$^{-/-}$ + LPS groups [Figure 4a-d].

Superoxide levels in the organs of wild-type or STE20/SPS1-realted proline/alanine-rich kinase knockout mice treated with lipopolysaccharide

At 24 h after LPS, the levels of superoxide in the lung, liver, kidney, and aorta were slightly elevated in wild-type mice [Figure 5a-d]. Although SPAK$^{+/+}$ + LPS group had higher superoxide levels than SPAK$^{+/+}$ + vehicle group, it was not significant. Moreover, there were no significant differences in superoxide levels among SPAK$^{+/+}$ + LPS, SPAK$^{+/-}$ + LPS, and SPAK$^{-/-}$ + LPS groups [Figure 5a-d].

Survival rate in wild-type or STE20/SPS1-realted proline/alanine-rich kinase knockout mice treated with lipopolysaccharide

The body weight was not significantly different among all groups at the beginning of experiment [Table 1]. In the SPAK$^{+/+}$ + vehicle, SPAK$^{+/-}$ + vehicle, and SPAK$^{-/-}$ + vehicle groups, no mortality was observed within 24 h [Table 2]. The 15-h survival rates of SPAK$^{+/+}$ + LPS, SPAK$^{+/-}$ + LPS, and SPAK$^{-/-}$ + LPS groups were 89%, 94%, and 92%, respectively. The 18-h survival rates of SPAK$^{+/+}$ + LPS, SPAK$^{+/-}$ + LPS, and SPAK$^{-/-}$ + LPS groups were 78%, 75%, and 92%, respectively. The 24-h survival rates of SPAK$^{+/+}$ + LPS, SPAK$^{+/-}$ + LPS, and SPAK$^{-/-}$ + LPS groups were 67%, 75%, and 77%, respectively [Table 2].

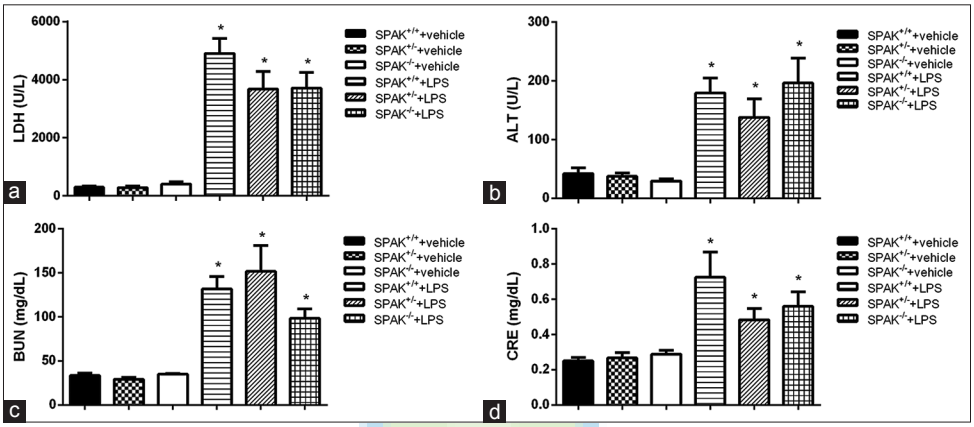

Figure 3: The changes of plasma (a) lactate dehydrogenase, (b) alanine aminotransferase, (c) blood urea nitrogen, and (d) creatinine at the end of experiment (at 24 h) in wild-type or SPAK knockout mice treated with lipopolysaccharide. Depicted are SPAK$^{+/+}$ mice that received saline (SPAK$^{+/+}$ + vehicle, n = 14) or lipopolysaccharide (SPAK$^{+/+}$ + lipopolysaccharide, n = 12), SPAK$^{+/-}$ mice that received saline (SPAK$^{+/-}$ + vehicle, n = 12) or lipopolysaccharide (SPAK$^{+/-}$ + lipopolysaccharide, n = 12), and SPAK$^{-/-}$ mice that received saline (SPAK$^{-/-}$ + vehicle, n = 8) or lipopolysaccharide (SPAK$^{-/-}$ + lipopolysaccharide, n = 10). Data are expressed as mean ± standard error of mean *P < 0.05, all versus SPAK$^{+/+}$ + vehicle mice; $^{\#}P$ < 0.05, without versus with SPAK in lipopolysaccharide mice. SPAK = STE20/SPS1-realted proline/alanine-rich kinase

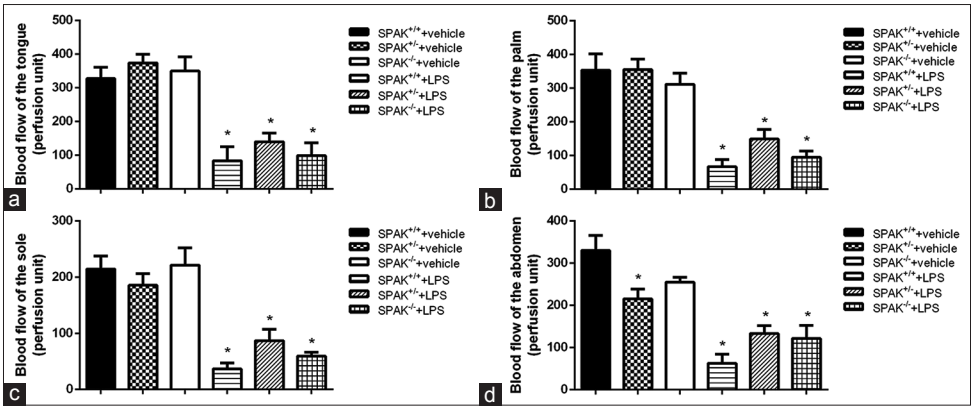

Figure 4: The changes of blood flow in (a) tongue, (b) palm, (c) sole, and (d) abdomen at the end of experiment (at 24 h) in wild-type or SPAK knockout mice treated with lipopolysaccharide. Depicted are SPAK$^{+/+}$ mice that received saline (SPAK$^{+/+}$ + vehicle, n = 10) or lipopolysaccharide (SPAK$^{+/+}$ + lipopolysaccharide, n = 9), SPAK$^{+/-}$mice that received saline (SPAK$^{+/-}$ + vehicle, n = 12) or lipopolysaccharide (SPAK$^{+/-}$ + lipopolysaccharide, n = 12), and SPAK$^{-/-}$ mice that received saline (SPAK$^{-/-}$ + vehicle, n = 5) or lipopolysaccharide (SPAK$^{-/-}$ + lipopolysaccharide, n = 7). Data are expressed as mean ± standard error of mean *P < 0.05, all versus SPAK$^{+/+}$ + vehicle mice; $^{\#}P$ < 0.05, without versus with SPAK in lipopolysaccharide mice. SPAK = STE20/SPS1-realted proline/alanine-rich kinase

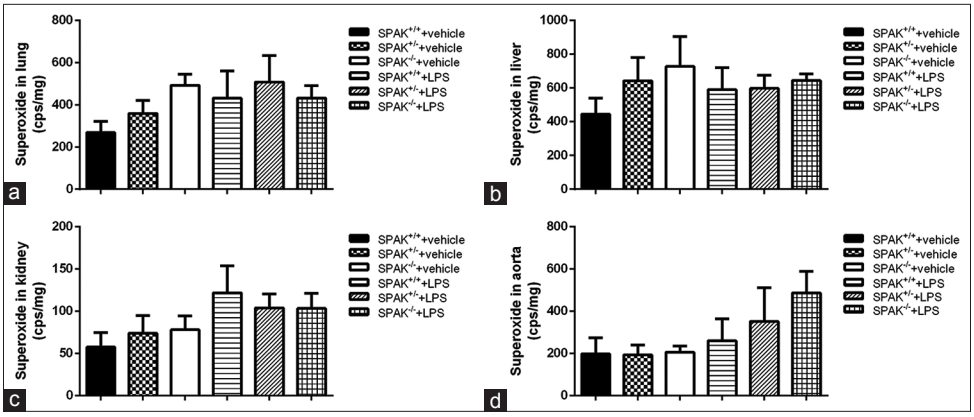

Figure 5: The changes of superoxide levels in (a) lung, (b) liver, (c) kidney, and (d) aorta at the end of experiment (at 24 h) in wild-type or SPAK knockout mice treated with lipopolysaccharide. Depicted are $SPAK^{+/+}$ mice that received saline ($SPAK^{+/+}$ + vehicle, n = 14) or lipopolysaccharide ($SPAK^{+/+}$ + lipopolysaccharide, n = 12), $SPAK^{+/-}$ mice that received saline ($SPAK^{+/-}$ + vehicle, n = 12) or lipopolysaccharide ($SPAK^{+/-}$ + lipopolysaccharide, n = 12), and $SPAK^{-/-}$ mice that received saline ($SPAK^{-/-}$ +vehicle, n = 8) or lipopolysaccharide ($SPAK^{-/-}$ + lipopolysaccharide, n = 10). Data are expressed as mean ± standard error of mean *$P < 0.05$, all versus $SPAK^{+/+}$ + vehicle mice; #$P < 0.05$, without versus with SPS1-realted proline/alanine-rich kinase in lipopolysaccharide mice. SPAK = STE20/SPS1-realted proline/alanine-rich kinase

Table 1: Body weight in wild-type and STE20/SPS1-realted proline/alanine-rich kinase knockout mice

Groups	Body weight (g)
$SPAK^{+/+}$ +vehicle	24.7±0.4
$SPAK^{+/-}$ +vehicle	24.5±0.4
$SPAK^{-/-}$ +vehicle	25.2±1.1
$SPAK^{+/+}$ + LPS	24.6±0.5
$SPAK^{+/-}$ + LPS	25.8±1.0
$SPAK^{-/-}$ + LPS	24.5±0.8

Depiction of the body weight in different groups of animals at the beginning of experiment. $SPAK^{+/+}$ + vehicle, $SPAK^{+/+}$ mice were given saline at time 0 (n=14); $SPAK^{+/-}$ + vehicle, $SPAK^{+/-}$ mice were given saline at time 0 (n=12); $SPAK^{-/-}$ + vehicle, $SPAK^{-/-}$ mice were given saline at time 0 (n=8); $SPAK^{+/+}$ + LPS, $SPAK^{+/+}$ mice were given LPS at time 0 (n=12); $SPAK^{+/-}$ + LPS, $SPAK^{+/-}$ mice were given LPS at time 0 (n=12); $SPAK^{-/-}$ + LPS, $SPAK^{-/-}$ mice were given LPS at time 0 (n=10). LPS = Lipopolysaccharide; SPAK = STE20/SPS1-realted proline/alanine-rich kinase

DISCUSSION

In this study, wild-type mice that received LPS showed circulatory failure, hypoglycemia, multiple organ dysfunction, and higher mortality, as seen in patients with septic shock. The NO levels of plasma were increased and blood flow of the tongue, palm, sole, and abdomen were decreased in wild-type mice with endotoxic shock. However, SPAK knockout mice treated with LPS did not show different phenotype regarding all these functional parameters when compared to the wild-type mice treated with LPS. Thus, these results indicate that SPAK does not contribute to the pathological responses of sepsis.

LPS is the main mediator of Gram-negative septic shock with high mortality rates. Administration of LPS to animals mimics the symptoms of septic shock, resulting in severe hypotension. Excessive formation of NO by inducible NO synthase (iNOS) has been proposed to be the major reason involved in vasodilatation and hypotension in sepsis.[15,16] Administration of NOS inhibitor in animals with septic shock has been demonstrated to restore blood pressure and increase survival rates.[15,17,18] In addition, the hypotension and high mortality caused by endotoxin are attenuated in iNOS-deficient mice.[19] Indeed, our study showed that LPS increased plasma NO levels and elicited severe hypotension in wild-type mice. However, SPAK knockout mice treated with LPS exhibited similar plasma NO levels and blood pressure when compared to the wild-type mice treated with LPS. These results indicate that SPAK is not a promising target to reverse abnormal plasma NO levels in endotoxemia.

It is known that reactive radicals are simultaneously produced by LPS-stimulated macrophages.[20] Massive production of ROS exacerbates the overwhelming inflammatory response and causes organ injury.[21,22] Furthermore, excessive formation of peroxynitrite from the reaction between NO and superoxide anion could prompt enormous oxidative injury and multiple organ dysfunction in sepsis.[23,24] Many studies have demonstrated that ROS scavenger or antioxidant therapy can diminish organ injury and increase the survival rate of animals with sepsis.[25,26] In view of these data, the imbalance in redox state is one of the critical triggers in multiple organ dysfunction in sepsis. We found that both wild-type and SPAK knockout mice treated with LPS displayed multiple organ dysfunction syndrome. There were no significant differences in superoxide levels and organ function parameters between wild-type and SPAK knockout mice with endotoxemia. Consequently, SPAK may not serve as a therapeutic target for treatment of organ dysfunction caused by oxidative stress.

Table 2: Changes of survival rate in wild-type or STE20/SPS1-realted proline/alanine-rich kinase knockout mice treated with lipopolysaccharide

Groups	9-h survival rate (%)	12-h survival rate (%)	15-h survival rate (%)	18-h survival rate (%)	21-h survival rate (%)	24-h survival rate (%)
$SPAK^{+/+}$ + vehicle	100 (14/14)	100 (14/14)	100 (14/14)	100 (14/14)	100 (14/14)	100 (14/14)
$SPAK^{+/-}$ + vehicle	100 (12/12)	100 (12/12)	100 (12/12)	100 (12/12)	100 (12/12)	100 (12/12)
$SPAK^{-/-}$ + vehicle	100 (8/8)	100 (8/8)	100 (8/8)	100 (8/8)	100 (8/8)	100 (8/8)
$SPAK^{+/+}$ + LPS	100 (18/18)	100 (18/18)	89 (16/18)	78 (14/18)	67 (12/18)	67 (12/18)
$SPAK^{+/-}$ + LPS	100 (16/16)	100 (16/16)	94 (15/16)	75 (12/16)	75 (12/16)	75 (12/16)
$SPAK^{-/-}$ + LPS	100 (13/13)	100 (13/13)	92 (12/13)	92 (12/13)	77 (10/13)	77 (10/13)

Depicted is the survival rate in different groups of animals during the experimental period. $SPAK^{+/+}$ + vehicle, $SPAK^{+/+}$ mice were given saline at time 0 (n=14); $SPAK^{+/-}$ + vehicle, $SPAK^{+/-}$ mice were given saline at time 0 (n=12); $SPAK^{-/-}$ + vehicle, $SPAK^{-/-}$ mice were given saline at time 0 (n=8); $SPAK^{+/+}$ + LPS, $SPAK^{+/+}$ mice were given LPS at time 0 (n=18); $SPAK^{+/-}$ + LPS, $SPAK^{+/-}$ mice were given LPS at time 0 (n=16); $SPAK^{-/-}$ + LPS, $SPAK^{-/-}$ mice were given LPS at time 0 (n=13). LPS = Lipopolysaccharide; SPAK = STE20/SPS1-realted proline/alanine-rich kinase

Microcirculation is a crucial system responsible for the delivery of oxygen to the organs of the body. Microvascular dysfunction reduces perfusion and results in tissue hypoxia. Microvascular blood flow is significantly diminished in patients with severe sepsis and related to the worse outcome of patients.[27,28] Adequate fluid resuscitation and cardiovascular support are used to restore microcirculatory flow, tissue perfusion, and organ function in sepsis.[24,29] In this study, the density of blood flow was significantly reduced in mice with endotoxic shock. However, no significant difference in blood flow was observed between wild-type and SPAK knockout mice treated with LPS. Therefore, SPAK may not be associated with the improvement in microvascular dysfunction of endotoxemia.

Despite we use SPAK knockout mice in this experimental research, one of the major concerns is functional compensation. There are some upstream proteins involved in the regulation of NKCC phosphorylation, such as With-No-Lysin [K] kinase (WNK) and oxidative-stress-responsive kinase 1 (OSR1). These molecules may compensate SPAK functions in SPAK knockout mice. However, no highly selective inhibitors of SPAK, WNK or OSR1 have been discovered. The results of SPAK inhibitors against sepsis may differ from this study.

CONCLUSION

Our results showed that inhibition of SPAK did not improve circulatory failure, hypoglycemia, multiple organ dysfunction or higher mortality in mice that received LPS. Thus, we suggest that SPAK does not play an important role in the management of endotoxic shock, at least. However, further studies are needed to confirm these data in other models of sepsis.

Acknowledgments

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Nil.

Conflicts of interest

There are no conflicts of interest.

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