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Deficiency of Smad3 results in enhanced inducible nitric oxide synthase–mediated hypotension in lipopolysaccharide-induced endotoxemia

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ABSTRACT

Background: Smad3 is a principal intracellular mediator of signaling for transforming growth factor β, a cytokine involved in pleiotropic pathophysiological processes including inflammation and immunity. The function of Smad3 in regulating inducible nitric oxide synthase (iNOS) expression and septic shock has not been characterized.

Methods: Smad3$^{-/-}$(referred hereafter as KO) and wild-type (WT) mice were injected intraperitoneally with lipopolysaccharide (LPS) to induce the septic hypotension. Mortality, blood pressure, and plasma levels of nitrite were measured. The iNOS messenger RNA and protein levels in lung, kidney, and spleen were also analyzed.

Results: Mice lacking functional Smad3 respond to LPS with greater mortality than their WT littermates. The high mortality of KO mice is accompanied by enhanced hypotension after intraperitoneal injection of LPS. Both KO and WT mice displayed an increase in plasma nitrite during the experimental period; however, LPS administration caused more dramatic changes in KO mice than WT mice. Likewise, the iNOS messenger RNA and protein levels in lung, kidney, and spleen were more strongly increased in KO mice than in WT mice after LPS administration.

Conclusions: Defects in the *Smad3* gene may increase susceptibility to the development of septic hypotension because of enhanced iNOS production.

1. Introduction

Septic shock is characterized by hypotension, hyporeactivity to vasoconstricting agents, inadequate tissue perfusion, vascular damage, and disseminated intravascular coagulation leading to multiple organ failure and death [1]. Systemic hypotension during bacterial sepsis is associated with the excessive production of nitric oxide (NO). Persistent and excessive NO production by inducible nitric oxide synthase (iNOS) relaxes the vascular smooth muscle and decreases its

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responsiveness to vasoconstrictive agents, such as norepinephrine, resulting in septic hypotension [2]. This is accompanied by increased levels of the stable metabolites of NO—nitrite (NO^-_2) and nitrate (NO^-_3)—in the plasma of septic patients [3] and mice [4]. Several cell signaling pathways, including mitogen-activated protein kinase and nuclear factor κB pathways, have been reported to regulate endotoxin-associated iNOS expression [5]; however, whether transforming growth factor β (TGF-β)/Smad signaling plays a role in the development of hypotension remains unanswered.

Smad3 is an intracellular mediator of TGF-β, a pleiotropic cytokine involved in many pathophysiological processes including inflammation and immunity [6]. The activated heteromeric complex of TGF-β type I and type II transmembrane serine-threonine kinase receptors induces phosphorylation of Smad3 and Smad2, which form hetero-oligomeric complexes with Smad4. The complexes then translocate to the nucleus and regulate transcriptional responses together with DNA binding cofactors. Smad3 null mice generated in different laboratories [7,8] remain viable and fertile. Therefore, $Smad3^{-/-}$ (referred hereafter as KO) mice provide an appropriate model for studying the function of the TGF-β1/Smad pathway *in vivo*.

TGF-β1 has a well-established role in inhibiting iNOS expression *in vitro* and *in vivo* [9,10]. However, the function of Smad3 in regulating iNOS expression has not been characterized. We hypothesized that Smad3 may be involved in the regulation of iNOS in sepsis, consequently regulating hypotension and septic shock in a mouse model.

2. Materials and methods

2.1. Mice

The generation of $Smad3^{ex8/ex8}$ null mice by homologous recombination and genotype determination by polymerase chain reaction have been described [7]. Smad3 heterozygote breeding pairs were generously provided by Professor Xiao Yang (Academy of Medical Sciences of Chinese PLA, Beijing, China). Mice heterozygous for the targeted disruption of *Smad3* were crossbred to produce homozygous offspring and housed under pathogen-free conditions in the animal facility of the Second Military Medical University. All mice were maintained at 24°C with a relative humidity between 30% and 70% on a 12-h:12-h light–dark cycle. Mice were fed an irradiated diet *ad libitum*. All animals received humane care in accordance with the guidelines of the National Institutes of Health under a protocol approved by the Ethics Committee of the Second Military Medical University in Shanghai.

2.2. Endotoxic shock model

For studies of endotoxic shock model, 8-wk-old male mice were used. KO and the wild-type (WT) $Smad3^{+/+}$ littermates were randomly grouped into lipopolysaccharide (LPS) group and control group. Their temperature, respiratory frequency, and heart rate were carefully observed to make sure that each mice had an equivalent physiological baseline before LPS administration. Mice were starved, but allowed free access to water for 24 h before experimentation. For LPS responses, mice were injected intraperitoneally (i.p.) with 10.0 mg LPS/kg body weight. LPS from *Escherichia coli* was obtained from Sigma–Aldrich Co (026:B6 L2654, 0111:B4 L4391, 0127:B8 L4516, 055:B5 L6529). Mice were anesthetized with pentobarbital sodium (40 mg/kg, i.p.) for blood pressure measurements at 0, 3, 6, 9, and 12 h (four mice at each time point) after LPS administration. Mice were euthanized, blood was collected by cardiac puncture for plasma, and tissue samples from lung, kidney, and spleen were collected and frozen at −80°C. To study the protective effect of Smad3 protein, eight KO and WT mice were injected i.p. with a lethal dose of 15.0 mg LPS/kg body weight. The mice were observed for 24 h, and survival rates were calculated [11].

2.3. Blood pressure measurements

Animals were anesthetized with pentobarbital sodium (40 mg/kg, i.p.) 30 min before blood pressure measurement. The carotid arteries were then isolated, and 6-0 prolene sutures were placed around them. The distal ties were secured, a 32-gauge needle was used to pierce the arteries, and PE-50 tubing (Clay-Adams, Parsippany, NJ) was inserted into the carotid arteries. The proximal sutures were then secured, and catheters were connected to a blood pressure transducer. Blood pressure was continuously monitored for 10 min using the MPA-Cardiac Function Acquisition and Analysis System (Shanghai Alcott Biotech Co, Ltd, Shanghai, China) with a Lenovo desktop computer (ThinkCentre M8000s, Lenovo, China).

2.4. Preparation of cytoplasmic extracts

After the blood pressure measurements, mice were euthanized and the lungs, kidneys, and spleens were removed and frozen at −80°C for further analyses. The organs were homogenized as described [12]. Protein concentrations were measured using the bicinchoninic acid Protein Assay Reagent kit (Pierce, Rockford, IL) with bovine serum albumin as a standard.

2.5. Measurement of plasma nitrite levels

Concentrations of nitrite in plasma were measured using the diazotization method based on the Griess reaction according to the NO assay kit instructions (catalog no. S0021; Beyotime Biotechnology Co, Jiangsu, China). Nitrite concentrations in the plasma were calculated by linear regression analyses using standard calibration curves of sodium nitrite.

2.6. RNA isolation and real-time quantitative reverse transcription–polymerase chain reaction

Total RNA was isolated from lung, kidney, and spleen tissue samples of KO and WT mice using a TRIzol reagent (Invitrogen, Gaithersburg, MD). Relative quantification of the gene expression was performed by a two-step real-time polymerase chain reaction using complementary DNA as a template and SYBR Premix Ex TaqTM (Takara Biotechnology Company, Dalian, China) on a LightCycler system (Roche Molecular Biochemicals, IN). Data analysis was performed using LightCycler software. The $2^{-\Delta\Delta Ct}$ method was used to