Original Paper

Pretreatment of Sialic Acid Efficiently Prevents Lipopolysaccharide-Induced Acute Renal Failure and Suppresses TLR4/gp91-Mediated Apoptotic Signaling

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Key Words

Lipopolysaccharide • Sialic acid • Reactive oxygen species • Apoptosis • Gp91 • Toll-like receptors

Abstract

Background/Aims: Lipopolysaccharides (LPS) binding to Toll-like receptor 4 (TLR4) activate NADPH oxidase gp91 subunit-mediated inflammation and oxidative damage. Recognizing the high binding affinity of sialic acid (SA) with LPS, we further explored the preventive potential of SA pretreatment on LPS-evoked acute renal failure (ARF). **Methods:** We determined the effect of intravenous SA 30 min before LPS-induced injury in urethane-anesthetized female Wistar rats by evaluating kidney reactive oxygen species (ROS) responses, renal and systemic hemodynamics, renal function, histopathology, and molecular mechanisms. **Results:** LPS time-dependently reduced arterial blood pressure, renal microcirculation, and increased blood urea nitrogen and creatinine in the rats. LPS enhanced monocyte/macrophage infiltration and ROS production, and subsequently impaired kidneys with the enhancement of TLR4/NADPH oxidase gp91/Caspase 3/poly-(ADP-ribose)-polymerase (PARP)-mediated apoptosis in the kidneys. SA pretreatment effectively alleviated LPS-induced ARF. The levels of LPS-increased ED-1 infiltration and ROS production in the kidney were significantly depressed by SA pretreatment. Furthermore, SA pretreatment significantly depressed TLR4 activation, gp91 expression, and Caspase 3/PARP induced apoptosis in the kidneys. **Conclusion:** We suggest that pretreatment of SA significantly and preventively attenuated LPS-induced detrimental effects on systemic and renal hemodynamics, renal ROS production and renal function, as well as, LPS-activated TLR4/gp91/Caspase3 mediated apoptosis signaling.

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Introduction

Sepsis, a major medical problem commonly leading to multiple organ failure, is one of the main causes of death in critical care medicine. The elderly are predisposed to sepsis, severe sepsis and septic shock due to multiple co-morbidities, repeated and prolonged hospitalizations, impaired immunity, functional limitations, and as well, the effects of aging itself [1]. Sepsis may be present in nearly all patients requiring hospitalization with an infection, while severe sepsis is present in a half to three-quarters of critically ill patients [2]. Therefore, strategies for preventing sepsis and subsequent progress are important and worthy of exploration.

The development of sepsis into organ failure involves inflammatory cytokines and reactive oxygen species (ROS) production, which may be caused by the migration of leukocytes, lymphocytes, and platelets to the infected areas and consequently resulted in local blood flow reduction and ischemia/reperfusion injury [3, 4]. On entering the blood stream, lipopolysaccharides (LPS), an endotoxin, may cause septic reactions with a variety of symptoms such as systemic inflammation and hypotension in the animals and human [4, 5]. Acute renal failure is a common complication in septic patients [6, 7] possibly by exacerbated ROS-evoked abnormal signal transduction, inflammatory monocyte/macrophage infiltration, cellular dysfunction and death in the kidney [8-10]. Non-programmed necrotic cell death (necrosis) and three types of programmed cell death, apoptosis, autophagy, and pyroptosis may play important roles in LPS-induced acute renal failure [11, 12]. ROS generation from the NADPH oxidase may induce apoptosis by the activation of caspases, lysosomal proteases, or endonucleases [8, 12-15]. The increased ROS trigger apoptosis by activating Bax expression/caspase 3 activity/poly-(ADP-ribose)-polymerase (PARP) fragments [8, 14, 15]. The inhibition of apoptosis [16] is an important target for reducing LPS-induced acute renal failure [12].

The key principles of current clinical guidelines for the treatment of patients with established sepsis [17], regardless of Intensive Care Unit admission, include early quantitative oxygen and fluid resuscitation, prompt laboratory and imaging studies for confirmation of infection source(s), early administration of appropriately empirical or broad-spectrum antimicrobials therapy, and inotropic agents or vasopressors if needed. Besides, removing circulating endotoxin by adsorption [18], polymyxin B-immobilized fiber column hemoperfusion has also been reported to be effective and used for patients with sepsis or septic shock, especially in Japan and parts of Western Europe [19-22]. However, direct systemic administration of polymyxin B is associated with risks of neurotoxic and nephrotoxic adverse effects. On the other hand, our previous data found that the exogenous sialic acid (SA), binding to lipid A, LPS and *Helicobacter pylori*, can neutralize the toxicity and subsequently ameliorate LPS-induced tissue/organ injury [4].

N-acetylneuraminic acid, the most common SA, majorly found on the cell surface glycoproteins and glycolipids [23], and participated in E-selectin binding [23], can be extracted from the edible bird's nest made of the saliva of *Collocalia* sp. traditionally used in ancient China [24]. Extract of the edible bird's nest stimulates the growth factor for epidermal tissue resulting the repairing of cells [25, 26], and strongly inhibits infection with influenza viruses and hemagglutination of influenza viruses to erythrocytes [24]. In other words, edible bird's nest is a safe and valid natural source for the prevention of virus infection [27], because SA affects the adherence of influenza virus particles to their target cells [28].

We have recently reported that LPS activated major TLR4/NADPH oxidase gp91 signaling to evoke apoptosis in the kidney [12]. In the present study, we further explored whether pretreatment of antioxidant and anti-inflammatory SA prevents acute renal injury through suppressing TLR4/NADPH oxidase/ROS mediated apoptosis signaling.

Materials and Methods

Surgery and animal preparation

Female Wistar rats (220–250 g) were purchased from BioLASCO Taiwan Co. Ltd. (Taipei) and housed at the Experimental Animal Center, National Taiwan Normal University, at a constant temperature and with a consistent light cycle (light from 0700 to 1800 h). All the surgical and experimental procedures were approved by the Institutional Animal Care and Use Committee of the National Taiwan Normal University and were in accordance with the guidelines of the National Science Council of Republic of China (NSC 1997). All efforts were made to minimize animal suffering and the number of animals used throughout the experiment.

On the experimental day, the rats were anesthetized with subcutaneous urethane (1.2 g/kg). The body temperature was kept at 37.0°C by an infrared light and was detected by a rectal thermometer. The surgical procedures were detailed described previously [12].

In this study, we used LPS (*Escherichia coli* 0127: B8, Sigma-Aldrich, St. Louis, MO) intravenously injected to the rats through the femoral vein to induce endotoxemia and acute renal injury. Totally, eighteen rats were divided into three groups: normal sham group was given saline (n=6); LPS group was given a dose of LPS (50 mg/kg, iv, n=6); SA+LPS rats were challenged with LPS (50 mg/kg, iv, n=6) plus SA pretreatment (10 mg/kg, iv) thirty min before LPS treatment. We determined the ROS level in the kidney and several biochemical parameters including blood urea nitrogen (BUN) and creatinine, using commercially available analytical kits (Sigma, St. Louis, MO) as described previously [12].

The rats were sacrificed with an intravenous KCl (0.1 mg/mL) six hours after administration of LPS. The kidneys were immediately removed and divided into two parts. One part was stored in 10% neutral buffered formalin for immunocytochemic assay, and the other was frozen in liquid nitrogen and stored at –70°C for protein isolation or other assay.

Renal microcirculation measurement

The real-time effects of SA pretreatment on LPS-induced change in renal hemodynamics were examined with a full-field laser perfusion imager as described previously [12]. The measured parameter for quantitation of renal microcirculation was displayed with perfusion unit from the kidney surface.

In vivo chemiluminescence recording of ROS activity

The ROS response to LPS toxicity was directly determined from the kidney by intrarenal arterial infusion of a superoxide anion probe, 2-Methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo-[1,2-a]-pyrazin-3-one-hydrochloride (MCLA) (0.2 mg/mL/h, TCI-Ace, Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan) by using a Chemiluminescence Analyzing System (CLD-110, Tohoku Electronic In. Co., Sendai, Japan) as described previously [9].

Plasma endotoxin and SA level assay

The endotoxin levels for in vitro LPS samples were quantified for their Limulus amebocyte lysate activity by using the chromogenic Limulus amebocyte lysate end point assay kit (Lonza Walkersville Inc., MD) according to the manufacturer's instruction. The pyrogen-free water supplied with the kit was used to reconstitute the endotoxin standard (*E. coli* 0111:B4). Serial dilutions of LPS samples were made to bring their concentrations into the range of the standard curve. Absorbance values of blank and endotoxin activity values of standards and test samples were measured according to directions of Lonza for this kit. SA was determined by a standard procedure of a SA quantitation kit (Sigma-Aldrich) [4].

In situ demonstration of oxidative stress and apoptosis formation

We performed ED-1 (an IgG1 murine clone directed against the rat pan-macrophage surface protein CD68) mediated inflammation and terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) apoptosis method [12] in the kidney subjected to LPS-induced renal injury. The renal sections (5 µm) were prepared, deparaffinized, and stained by each stain. For renal macrophage (ED-1) staining as an inflammatory marker, these sections were incubated overnight at 4°C with a mouse anti-rat antibody to ED-1 (CD68, 1:200, Serotec, Sydney, NSW, Australia). A biotinylated secondary antibody (Dako, Botany, NSW, Australia) was then applied followed by streptavidin conjugated to HRP (Dako). The chromogen used was Dako Liquid diaminobenzene (DAB). Twenty high-power (×400) fields were randomly selected for

each section, and the value of each oxidative stress was analyzed using a Sonix Image Setup (Sonix Technology Co., Ltd) containing image analyzing software Carl Zeiss AxioVision Rel.4.8.2 (Future Optics & Tech. Co. Ltd., Hangzhou, China).

TLR4, gp91 and Apoptosis-related proteins expression by Western blotting

The expression levels of TLR4, gp91, apoptosis-related proteins including caspase 3 and PARP were analyzed by Western blotting in kidney tissues from LPS rats with or without SA pretreatment. Western blotting method has been described elsewhere [12]. Antibodies raised against the activation fragments (32 kDa of proenzyme and 17 kDa of cleaved product) of caspase 3 (CPP32/Yama/Apopain, Upstate Biotechnology, Lake Placid, NY), gp91phox (NOX2, Abcam), TLR4 (rabbit anti-rat TLR4 antibody, Santa Cruz biotechnology Inc., CA), PARP (Cell Signaling Technology, Inc.), and β -actin (Sigma, Saint Louis, MI) were used. The density of the band with the appropriate molecular mass was determined semi-quantitatively by densitometry using an image analyzing system (Alpha Innotech, San Leandro, CA).

Statistical analysis

All values were expressed as mean $\pm$ standard error of the mean (SEM). Differences within groups were evaluated by paired t-test. One-way analysis of variance was used for establishing differences among groups. Intergroup comparisons were made by Duncan's multiple-range test. Differences were designated as significant if $P < 0.05$ was obtained.

Results

Premixing SA plus LPS evidenced the binding interaction between SA and LPS.

As shown in Figure 1, the in vitro study evidenced that SA interacted with LPS with the decreased concentration in respective SA concentration (Figure 1A) or LPS concentration (Figure 1B). Premixing SA plus LPS at the ratio of approximately 1:1 depressed the SA or LPS concentration.

SA pretreatment attenuated LPS-induced systemic hypotension

LPS significantly decreased arterial blood pressure 75 min after LPS stimulation and the arterial blood pressure was further reduced 6 h after LPS stimulation (Figure 2). SA pretreatment 30 min before LPS administration effectively prevented the critical drop in arterial blood pressure.

SA pretreatment attenuated LPS-depressing renal microcirculation

LPS significantly ($P < 0.05$) decreased the level of renal microcirculation 3 h after LPS stimulation (Figure 3A) in the rats, compared to respective controls. SA pretreatment significantly prevented the reduction in the renal microcirculation, determined by Moor flow image analysis, as in the LPS treated group (Figure 3B).

Fig. 1. The effect of premixing SA plus LPS on respective SA concentration (A) and LPS concentration (B) in in vitro study. N=3 in each test. 0, 0 mg/dL; 1, 1 mg/dL; 10, 10 mg/dL; ND, not detected.

Fig. 2. Effect of SA pretreatment on LPS-induced decreases in arterial blood pressure in the rats. The mean changes of mean arterial blood pressure were continuously recorded during 6 h of LPS treatment. * $P < 0.05$, vs. the matched time-course point of the control group without LPS and SA treatment. # $P < 0.05$, vs. the matched time-course point of the LPS group.

Fig. 3. Effect of SA pretreatment on LPS-induced decreases in microvascular blood flow in the kidneys. LPS gradually reduced microvascular blood flow in the kidney after 3 and 6 h (A). An increase in perfusion units toward red colors and a decrease in perfusion units toward blue colors are indicated. The black-gray colors shown in A are indeed false colors in the LPS treated kidney. During 6 h of LPS stimulation, the changes in percentage of perfusion units of renal microcirculation of each group are depicted in B. Sham, control rats without LPS and SA treatment; LPS, the rats treated with iv 50 mg/kg LPS; SA+LPS, the rats pretreated with SA 10 mg/kg body weight iv at 30 min before LPS stimulation. * $P < 0.05$, vs. the Sham group without LPS and SA treatment. # $P < 0.05$, SA+LPS group vs. LPS group.

SA pretreatment reduced LPS-induced renal ROS amount

We determined the effect of SA pretreatment on LPS-induced oxidative stress in the kidney of the rats. The results showed that SA pretreatment per se did not increase renal ROS (Figure 4A). LPS increased the ROS levels of kidney *in vivo* time-dependently, and significantly ($P < 0.05$) 6 h after LPS treatment when compared to the value of the control group (Figure 4B). SA pretreatment significantly depressed the LPS-enhanced renal ROS (Figure 4B).

SA pretreatment effectively attenuated pathologic alteration, inflammation, and apoptosis in the LPS-treated kidneys

SA pretreatment per se did not induce apparent changes in histologic structures, inflammation and TUNEL-apoptosis in the rat kidneys without LPS stimulation (Figure 5). LPS treatment significantly increased tubular pathologic scores with H&E stain (Figure 5C),

inflammation marker ED-1 positive (monocyte/macrophage) stains (Figure 5G), and TUNEL-apoptosis (Figure 5K) stains, in contrast to sham controls (Figures 5A, 5E, 5I) and SA treated controls (Figures 5B, 5F, 5J). The degrees of LPS-enhanced pathologic alteration, ED-1, and TUNEL stains were markedly depressed by SA pretreatment (Figures 5D, 5H, 5L).

SA pretreatment decreased LPS induced TLR4/gp91/apoptosis signaling in the kidney

We determined the effect of SA pretreatment on TLR4/NADPH oxidase gp91-mediated oxidative stress/apoptosis in the LPS-treated kidneys (Figure 6). Original data of TLR4, gp91, and apoptosis-related caspase 3 and PARP are shown in Figure 6A. The expression of TLR4 (Figure 6B), gp91 (Figure 6C), caspase 3 (Figure 6D) and PARP (Figure 6E) were significantly enhanced in the LPS-treated kidneys when compared to the control group. On the other hand, the enhanced expression of TLR4, gp91, caspase 3 and PARP in the LPS-treated kidneys were significantly inhibited by SA pretreatment. These data infer that SA pretreatment has a potential preventive effect to reduce LPS-induced oxidative stress and apoptosis formation in the kidney.

SA pretreatment effectively prevented LPS-induced renal dysfunction

LPS significantly increased plasma BUN and creatinine 6 h after LPS treatment, whereas SA pretreatment significantly prevented the increases in plasma BUN and creatinine. The findings indicate SA pretreatment preventively and effectively alleviated LPS-induced acute renal injury (Figure 7).

Discussion

While our previous reports [4, 12] have presented the safety of oral intake of SA, the present study further demonstrate that intravenous administration of SA was safe and did not evoke any renal toxicity. In the present study, we further confirm SA pretreatment can effectively and preventively attenuate systemic hypotension, renal hypoperfusion, oxidative stress, inflammation, and apoptosis in acute renal injury induced by LPS.

Fig. 4. Effect of SA pretreatment on LPS-induced ROS *in vivo* in the kidneys. The original data of kidney ROS (A) and the statistic data of kidney ROS (B) show significant increases at the time after 6 h of LPS stimulation. Given 30 min before LPS treatment, SA at a dosage of 10 mg/kg body weight significantly decreased the ROS levels. * $P < 0.05$, vs. the control group without LPS and SA treatment. # $P < 0.05$, vs. LPS treatment.

Fig. 5. Effects of SA pretreatment on LPS-induced renal injuries. Pretreatment of SA per se did not evoke histologic structural changes (**B**), ED-1 infiltration (brown color; **F**) and TUNEL formation (brown color; **J**) when compared with control (Con) group. LPS induced marked histologic changes (**C**), ED-1 infiltration (**G**), and TUNEL-apoptosis formation (**K**) in the kidneys when compared to respective control sections (**A**, **E**, **I**). Intravenous SA pretreatment at 10 mg/kg body weight markedly improved LPS-induced pathologic parameters (**D**), ED-1 infiltration (**H**) and TUNEL number (**L**). These graphs are in 400 × amplification.

LPS release from Gram-negative bacteria or Gram-positive bacteria would activate TLR2 and TLR4 signaling to trigger release of inflammatory cytokines and inflammation, eventually leading to endotoxemia and sepsis [29]. LPS through TLR4 activating signaling is one of the major pathways for induction of endotoxemia and sepsis. Our study evidenced that LPS through TLR4/gp91/caspase 3/PARP signaling induced inflammation, hypotension, and acute renal failure in our animal model. The findings that SA pretreatment via neutralizing LPS toxicity effectively blocked LPS/TLR4/gp91 signaling suggest its promising preventive effect on LPS-induced sepsis.

We found that LPS toxicity decreased arterial blood pressure and renal microcirculation, subsequently resulting in ischemia/hypotension-mediated oxidative stress in the kidneys. Increased renal ROS *in vivo* was demonstrated by a chemiluminescent amplification technique and increased renal NADPH oxidase gp91 expression by western blot. Besides, the oxidative stress in the LPS-treated kidney was associated with a large amount of ED-1 macrophages/monocytes infiltration and TUNEL apoptotic cells in the kidney. Our previous report [12] stated that during endotoxemic acute renal failure, superoxide dismutase activity was decreased in the kidney, leading to the increase in O_2^- amount, the decrease

Fig. 6. Effects of SA pretreatment on LPS induced TLR4, gp91, and apoptosis-related proteins (Casp 3) and PARP expression. Original data of TLR4, gp91, Casp 3 and PARP expressions are demonstrated in A. The statistic data are indicated in B-E, respectively. Pretreatment of SA significantly attenuated LPS-enhanced TLR4, gp91, Casp 3 and PARP expressions, respectively. * $P < 0.05$, vs. the control (Con) group without LPS and SA treatment. # $P < 0.05$, SA+LPS group vs. LPS group.

in vascular NO bioavailability, and the severity in renal ischemic injury. In this study, we confirm the increase in the expression of Bax/caspase 3/PARP-apoptotic signaling and TLR4/gp91/ROS signaling upon LPS stimulation *in vivo*. The increased apoptosis formation followed by the increased oxidative stress amount has been found in ischemia/reperfusion, unilateral ureteral obstruction kidney and LPS-induced acute renal failure [8, 12, 30]. The possible signaling cascade for LPS toxicity (the cause) induced acute renal failure (the outcome effect) is through TLR4 activation to trigger gp91 mediated ROS production, and subsequently to evoke caspase 3-dependent PARP and apoptosis expression. Our data further show that the destruction of renal structures including tubular apoptotic cell death, and leukocyte infiltration and accumulation may also contribute to the acute renal failure. Our *in vitro* data showed that premixing SA plus LPS markedly decreased the concentration in SA by an SA quantitation assay and in LPS by endotoxin assay clearly demonstrating their binding interaction with LPS and

Fig. 7. Effect of SA pretreatment on LPS induced renal dysfunction evaluated by blood urea nitrogen (BUN) and creatinine. Pretreatment of SA significantly attenuated LPS-enhanced BUN and creatinine levels. * $P < 0.05$ vs. the control (Con) group without LPS and SA treatment. # $P < 0.05$ SA+LPS group vs. LPS group.

masking LPS toxicity. These effects would effectively suppress LPS evoked TLR4 activation, gp91/ROS expression, caspase 3/PARP/apoptosis formation and eventually endothelial and renal dysfunction. Therefore, the use of SA pretreatment through high binding affinity with LPS to counteract LPS toxicity and consequently inhibits LPS-induced downstream signaling in oxidative stress and apoptosis formation.

In our previous study [12], SA post-treatment within 30 min of LPS injection can effectively attenuate LPS-induced three types of programmed cell death: apoptosis, autophagy and pyroptosis. SA via the action of enhancement of X-linked inhibitor of apoptosis protein directly or indirectly suppresses caspase 3 activation, resulting in the inhibition of apoptosis [14]. *Helicobacter pylori*, a Gram-negative bacterium capable of chronic colonization of the human stomach, infected the target organ via the structure of LPS or lipid A [31]. SA displayed a direct anti-adhesion effect to *Helicobacter pylori* binding to gastric cells [14, 15]. Furthermore, in the *Helicobacter pylori* infected mice, SA effectively decreased oxidative stress and inflammatory markers including inducible nitric oxide synthase, gp91, CD68, caspase-1/IL-1 β -mediated pyroptosis and Bax/caspase 3/PARP-evoked apoptosis in the gastric mucosa [14, 15]. In the present study, for the first time, we recognized the preventive potential of SA to effectively neutralize LPS-induced toxicity, apoptotic injury and acute renal failure. The novel findings imply that SA-rich foods like edible bird's nest [24], chalaza and egg-yolk membrane [32] or others may be one promising strategy to treat the patients predisposing to sepsis, preventing its development and progression.

TLRs through TLR4 recognizes molecules derived from microbes and triggers critical roles in sepsis and inflammation [33]. Our previous study confirmed that LPS activate renal expression of TLR4, not TLR2, to induce acute renal failure [12]. Activation of the TLR4 by LPS increases inflammatory cytokines and chemokines production [34] and activates the NF- κ B pathway which has been implicated in the regulation of multiple biological phenomena including apoptosis [35]. Potent TLR4 stimulation produces severe reactions in the host, leading to multiple organ failure and death [36]. After LPS/TLR4 activation, NADPH oxidase gp91 is the primary source, whereas mitochondria is the secondary source of ROS production that contributes to elevated ROS levels in macrophages (ED-1) [37]. *In vivo* studies showed LPS-induced inflammation and septic shock were significantly reduced by ablation of NADPH oxidase gp91 [37]. Prevention of acute kidney injury in a rodent model of cirrhosis following selective gut decontamination is associated with reduced renal TLR4 expression [38]. In the present study, our results indicated that SA pretreatment effectively attenuated acute kidney injury and severe hypotension by the depressed renal TLR4 and gp91 expression because of high binding affinity between SA and LPS demonstrated previously [12]. SA depressed the number of inflammatory ED-1-positive cells also implying its anti-inflammation on immune cells.

We confirmed that SA could be used as a preventive strategy at earlier therapeutic stage [12] to counteract LPS toxicity and TLR4 activation. Our previous findings indicated that SA attenuates *Helicobacter pylori*-triggered epithelial caspase-1 activity and eradicates infection via its anti-adhesion effect [15]. In summary, our serial findings indicate that SA through pretreatment or early treatment can inhibit lipid A, endotoxin (LPS) and Gram negative bacteria-induced toxicity and infection.

Conclusion

SA pretreatment can counteract LPS-induced acute renal failure by its neutralization with LPS, antioxidant, anti-inflammation and anti-apoptotic mechanisms. Pretreatment of SA may attenuate LPS-induced acute renal failure via the depression of LPS/TLR4/gp91/ROS signaling and subsequent Caspase 3/PARP-mediated apoptosis in the kidneys.

Disclosure Statement

There are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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