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Blood–brain barrier dysfunction in mice induced by lipopolysaccharide is attenuated by dapsone

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ABSTRACT

Blood–brain barrier (BBB) dysfunction is a key event in the development of many central nervous system (CNS) diseases, such as septic encephalopathy and stroke. 4,4′-Diaminodiphenylsulfone (DDS, Dapsone) has displayed neuroprotective effect, but whether DDS has protective role on BBB integrity is not clear. This study was designed to examine the effect of DDS on lipopolysaccharide (LPS)-induced BBB disruption and oxidative stress in brain vessels. Using in vivo multiphoton imaging, we found that DDS administration significantly restored BBB integrity compromised by LPS. DDS also increased the expression of tight junction proteins occludin, zona occludens-1 (ZO-1) and claudin-5 in brain vessels. Level of reactive oxygen species (ROS) was reduced by DDS treatment, which may due to decreased nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and NOX2 expression. Our results showed that LPS-induced BBB dysfunction could be attenuated by DDS, indicated that DDS has a therapeutic potential for treating CNS infection and other BBB related diseases.

1. Introduction

The blood–brain barrier (BBB) is comprised of brain microvascular endothelial cells and regulates the permeation of molecules between the peripheral circulation and the central nervous system (CNS) [1]. The main structures responsible for the maintenance of the integrity and function of BBB are the intercellular tight junction proteins [2]. BBB dysfunction is considered to be an early and significant event in the pathogenesis of a variety of CNS diseases [3–5], so the protection of BBB integrity is regarded as one of the important issues for the treatment of many cerebral diseases [6].

Dysfunction of BBB can be induced by various extrinsic or intrinsic stimuli [7]. Such as sepsis, during which lipopolysaccharide (LPS) is released into circulation, promoting the generation of reactive oxygen species (ROS) in BBB [8]. A major sources of ROS during BBB dysfunction are nicotinamide adenine dinucleotide phosphate (NADPH) oxidases [9]. There are several members in the NADPH oxidase family, among which NOX2 containing NADPH oxidase is highly expressed in cerebral endothelium [10]. Reducing expression of NOX2 can protect mice from a variety of stimuli that produce cerebrovascular dysfunction [11–13].

4,4′-Diaminodiphenylsulfone (DDS, Dapsone) is currently used to treat leprosy [14] and is known to possess neuroprotective effect against ischemia, spinal cord injury and other brain damage [15–19]. One of its modes of action is anti-oxidant [17,20]. DDS reduces ROS generation in *Caenorhabditis elegans* thus extends its lifespan [20]. In non-phagocytic human diploid fibroblasts and a mouse lung injury model, DDS also suppresses ROS production by inhibiting the NOX system [21,22]. As BBB integrity is disrupted during the pathological process of many CNS disease, especially stroke, and DDS treatment reduces the infarction volume in rat ischemia model [18], so we hypothesize that DDS may have a protective role on BBB integrity, and may be via reducing oxidative stress status.

The present study focus on effect of DDS on LPS-induced BBB disruption in mice. Our data showed that DDS inhibited LPS-induced BBB dysfunction, restored expression of tight junction, as

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well as decreased ROS level, NOX2 expression and NADPH oxidase activity in brain vessels, indicated that DDS can protect the integrity of BBB during septic encephalopathy.

2. Materials and methods

2.1. Animals and drug administration

Female C57BL/6J mice (20–25 g body weight, 3 month-old) were housed under standard conditions in conventional cages and kept on standard chow diet and water ad libitum with 12-h light and dark cycles. The experiments were designed as follows: LPS (Sigma, St. Louis, MO) was dissolved in saline solution, and DDS (Sigma, St. Louis, MO) was dissolved in 4.5% polyethylene glycol in saline solution (PEG) [16]. Mice in control group were intraperitoneal (i.p.) injected with 100 μl saline solution, followed by 100 μl PEG after 30 min; mice in LPS group were injected with 1 mg/kg LPS (the volume was adjusted to 100 μl), followed by 100 μl PEG after 30 min; mice in LPS + DDS group were injected with 0.5, 2 or 5 mg/kg DDS (the volume was adjusted to 100 μl) 30 min after LPS administration. Assessments were performed 24 h after DDS (or PEG) injection. At least 6 mice per group were studied. All procedures were approved by the Animal Care Committee of Peking University Health Science Center in China.

2.2. Multiphoton in vivo microscopy analysis

In vivo multiphoton imaging was performed as previously described [3,23]. 24 h after LPS administration, mice were anesthetized and the cranium was firmly secured in a stereotaxic frame. A square cranial window was opened with a high-speed drill. Tetramethylrhodamine (TMR) -conjugated dextran (40 kDa, 0.1 mL of 10 mg/ml, Invitrogen, Carlsbad, CA) was injected via the tail vein. At the end of the experiment mice were killed by decapitation and brains were harvested, frozen and conserved at −80 °C until use.

In vivo images were acquired using a multiphoton microscope (Leica TCS SP5 MP, Chicago, IL) with 850 nm excitation and 20×/1.0 water immersion objective, 2 mm working distance. Once the area of interest was defined, 200 μm-thick stacks in the *Z*-axis (5 μm steps) were obtained with the Leica ASF software. The relative fluorescence intensity across cross-section of vessels was analyzed with ImageJ (NIH) software [24].

2.3. Isolation of brain capillaries

Brain capillaries were isolated using dextran gradient centrifugation as described [3,23]. The cortex and hippocampus were carefully dissected and the meninges were removed in ice-cold PBS containing 2% fetal bovine serum (FBS). The brain was homogenized and dextran (70 kDa, Pharmacia) was added to a concentration of 16%. The samples were then centrifuged at 6000*g* for 15 min. The capillary pellet located at the bottom of the tubes was collected and sequentially filtered through a 100 and 45 μm cell strainer. The capillaries remaining on top of the 45 μm cell strainer were collected in PBS.

2.4. Western blot analysis

Isolated brain capillaries were lysed in RIPA buffer, and western blot analysis was carried out as previously described [23].The protein concentration of each homogenate was determined using a BCA kit (Pierce). Extracts (60 μg of protein) were subjected to electrophoresis, and separated proteins were transferred onto PVDF membranes, which were then immunostained with the following primary antibodies against occludin (1:500, Invitrogen, Carlsbad, CA), claudin-5 (1:500, Abcam, San Diego, CA), ZO-1 (1:500, Invitrogen, Carlsbad, CA), NOX2 (1:3000, Santa Cruz Biotechnology, Santa Cruz, CA) and β-actin (1:5000, Sigma, St Louis, MO). The membranes were incubated with peroxidase-conjugated secondary antibodies, and immunoreactive bands were visualized with an ECL system.

2.5. Cerebrovascular oxidative stress assessment

Cerebrovascular oxidative stress was determined as described [23]. Isolated brain capillaries were incubated for 30 min in darkness in the presence of 10 μM dichlorofluorescein diacetate. Fluorescence intensity (λexc 485 nm, λem 530 nm) was read in a flexstation 3 microplate reader (molecular devices), normalized for protein concentration.

2.6. NADPH oxidase activity assay

NADPH oxidase activity was measured as described [11]. Isolated brain vessels were homogenized in Krebs–Ringer phosphate buffer at pH 7.4 (120 mmol/L NaCl, 4.8 mmol/L KCl, 1.2 mmol/L MgSO4, 2.2 mmol/L CaCl2, 0.1 mol/L phosphate buffer) with phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Roche). 5 μM lucigenin were added to tissue extracts. The reaction was initiated by adding 10 μL of NADPH solution to the final concentration 100 μM. For each sample, 30 s integrated chemiluminescence was measured and repeated for 5 times. After measurement, the samples were recollected and protein quantification was calculated using a BCA kit. Respective background counts were subtracted, and chemiluminescence was expressed in relative light units per microgram protein.

2.7. Statistical analysis

All data in the text and figures are expressed as mean ± SEM of at least three independent experiments [23,25]. A one-way analysis of variance (ANOVA) followed by Dunnett's or Tukey–Kramer's post hoc tests was performed to compare groups. Mean values were considered significantly different at $p < 0.05$, $p < 0.01$ or $p < 0.001$.

3. Results

3.1. BBB dysfunction induced by LPS was attenuated by DDS

To determine whether DDS has protective effect on BBB integrity, we examined the in vivo BBB permeability for TMR-dextran with multiphoton microscopy. We found that mice in control group displayed intact BBB, while LPS treatment increased BBB permeability significantly (Fig. 1A), the relative fluorescence intensity across a cross-section of vessels increased to 6.4 ± 0.7-fold in LPS group compared with control ($p < 0.01$) (Fig. 1B). Administration of 5 mg/kg DDS significantly inhibited LPS-induced BBB leakage (Fig. 1A), and the relative fluorescence intensity decreased to 1.6 ± 0.5-fold compared with control ($p < 0.01$) (Fig. 1B), suggested that DDS attenuated BBB injury during infection.

3.2. Expression of tight junction proteins was restored by DDS

BBB integrity is maintained by the presence of tight junction proteins, so we assessed expression of tight junction proteins ZO-1, occludin and claudin-5 in brain vessels of mice with western blotting. Results showed that LPS significantly decreased expression of ZO-1, occludin and claudin-5 to 0.5 ± 0.1-fold ($p < 0.001$),

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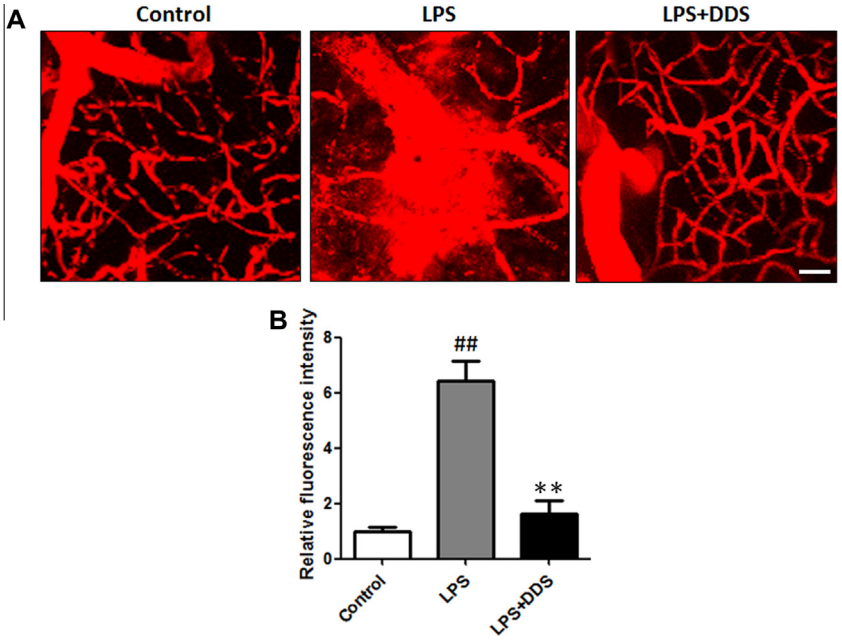

Fig. 1. BBB leakage induced by LPS was attenuated by DDS. (A) C57BL/6J mice were i.p. injected with vehicle (control), 1 mg/kg LPS (LPS) or 5 mg/kg DDS 30 min after LPS administration (LPS + DDS). After 24 h, TMR-dextran (MW = 40,000; red) was injected through tail vein, and the integrity of BBB was examined with in vivo time-lapse multiphoton imaging. Typical profile with $n = 6$ mice per group. Scale bar, 20 μm. (B) The relative fluorescence intensity across cross-section of vessels of each group. Data are means ± SEM ($^{\#\#}p < 0.01$ vs. control; $^{**}p < 0.01$ vs. LPS group). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

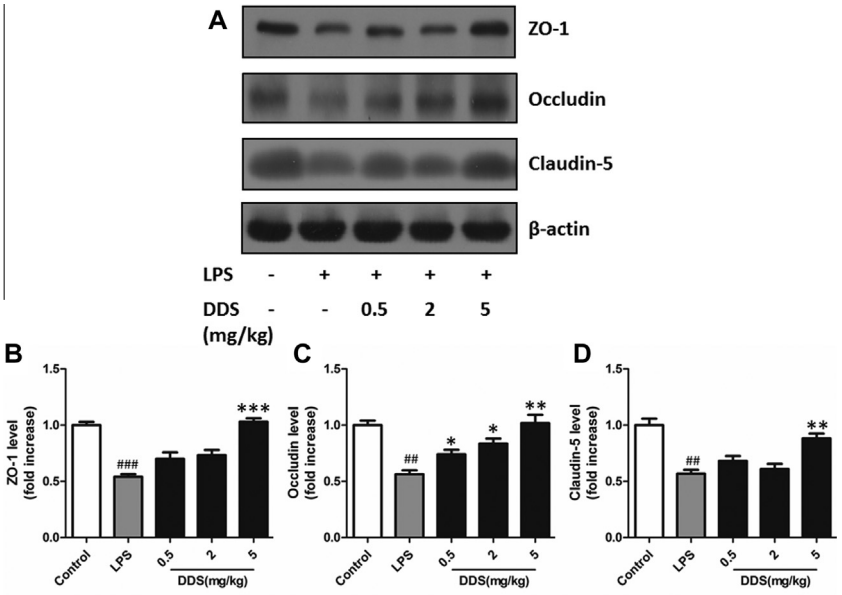

Fig. 2. Expression of tight junction proteins was restored by DDS. (A) C57BL/6J mice were i.p. injected with vehicle (control), 1 mg/kg LPS (LPS) or different concentrations of DDS (0.5, 2 or 5 mg/kg) 30 min after LPS administration, expression of tight junction proteins ZO-1, occludin, and claudin-5 in brain vessels were assessed with western blot ($n = 6$). β-Actin was used as loading control. Quantitative analysis of the expression levels of ZO-1 (B), occludin (C), and claudin-5 (D). Data are means ± SEM ($^{\#\#}p < 0.01$ vs. control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. LPS group).

0.6 ± 0.1-fold ($p < 0.01$) and 0.6 ± 0.1-fold ($p < 0.01$) respectively, compared with control (Fig. 2B–D). DDS treatment restored expression of tight junction proteins in a dose-dependent manner, for ZO-1 and claudin-5, 5 mg/kg DDS significantly increased their expression to 1.0 ± 0.1-fold ($p < 0.001$) and 0.9 ± 0.1-fold ($p < 0.01$) respectively, compared with control (Fig. 2B and D); for occludin, 0.5 mg/kg DDS already increased its expression to 0.7 ± 0.1-fold ($p < 0.05$) compared with control, and the expression also increased as dose of DDS increased, till at 5 mg/kg, expression of occludin elevated to 1.0 ± 0.1-fold ($p < 0.01$) compared with control (Fig. 2C). Together, these results indicated that DDS can restore the expression of tight junction proteins during infection.

3.3. LPS-induced ROS level was decreased by DDS

DDS has been known to display anti-oxidant effect in human diploid fibroblasts, neutrophils and *C. elegans* [20–22,26]. To determine whether the protective role of DDS at BBB is due to its

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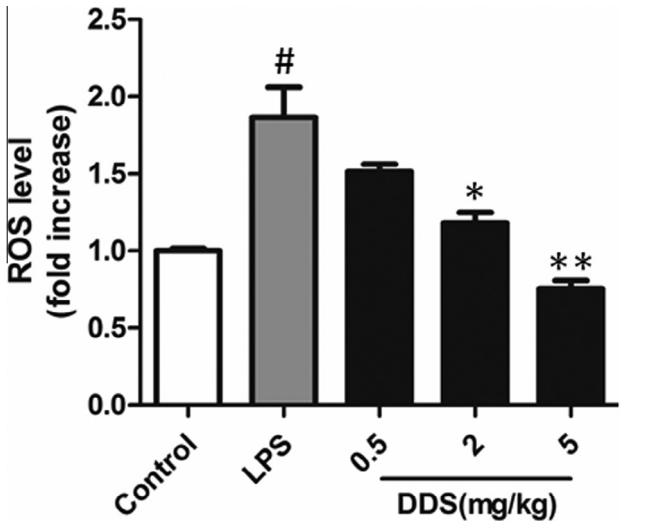

Fig. 3. LPS-induced ROS level was decreased by DDS. C57BL/6J mice were i.p. injected with vehicle (control), 1 mg/kg LPS (LPS) or different concentrations of DDS (0.5, 2 or 5 mg/kg) 30 min after LPS administration, level of ROS in brain vessels was measured with dichlorofluorescein diacetate assay ($n = 6$). Data are means ± SEM ($^{\#}p < 0.05$ vs. control; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. LPS group).

anti-oxidant property, we examined the level of ROS, which is the marker of oxidative stress status, in brain vessels. As shown in Fig. 3, LPS treatment significantly increased ROS level to 1.9 ± 0.2-fold ($p < 0.05$) compared with control, while administration of DDS decreased oxidative stress in a dose-dependent manner from 0.5, 2 to 5 mg/kg. At 2 and 5 mg/kg, level of ROS decreased significantly to 1.2 ± 0.1-fold ($p < 0.05$) and 0.8 ± 0.1-fold ($p < 0.01$) respectively, compared with control, indicated that the protective effect of DDS at BBB may via its ability to reduce ROS level.

3.4. LPS-induced NOX2 expression and NADPH oxidase activity were reduced by DDS

NOX2 containing NADPH oxidase is an important source of ROS in BBB [10]. We tested the effect of DDS on NOX2 expression. Results showed that LPS treatment increased NOX2 expression to 1.8 ± 0.1-fold ($p < 0.01$) compared with control, while DDS administration decreased NOX2 expression in a dose-dependent manner, at 2 and 5 mg/kg, level of NOX2 expression decreased significantly to 1.3 ± 0.1-fold ($p < 0.05$) and 1.2 ± 0.1-fold ($p < 0.05$) respectively, compared with control (Fig. 4A and B). Since NOX2 is the catalytic unit of NADPH oxidase, we further examined NADPH oxidase activity in brain vessels. Results showed that LPS treatment increased NADPH oxidase activity, while DDS administration decreased NADPH oxidase activity in a dose-dependent manner from 0.5, 2 to 5 mg/kg (Fig. 4C), which is consistent with the level of NOX2. These results indicated that effect of DDS on reducing ROS level may due to decreasing NOX2 expression and NADPH oxidase activity.

4. Discussion

Increased oxidative stress is very common during inflammatory response induced by systematic infection, and would lead to BBB leakage [24,27,28]. We have previously reported that in a chronic oxidative stress model, dietary supplementation of vitamin E significantly restored BBB integrity via reducing cerebrovascular oxidative stress level [23]. As dapsone is also an antioxidant and has displayed neuroprotective effects against ischemia, spinal cord injury and other brain damage [15–19]. We here investigate whether DDS can attenuate LPS-induced BBB dysfunction.

In the present work we observed that 1 mg/kg LPS administration increased BBB permeability of TMR-dextran (MW 40,000) from blood to brain parenchyma, which is in accordance with previous in vivo mice studies demonstrating the LPS-induced damage to the BBB integrity [24,29]. To assess the protective effect of DDS, mice were injected 5 mg/kg DDS 30 min after LPS administration, and we found that DDS treatment significantly reduced BBB permeability. BBB integrity is maintained by tight junction proteins, LPS regulates blood–brain barrier permeability by disrupting the tight junction [8]. Our results also showed that LPS decreased expression of tight junction proteins ZO-1, occludin and claudin-5 in brain vessels, while DDS restored the expression of tight junction proteins in a dose-dependent manner.

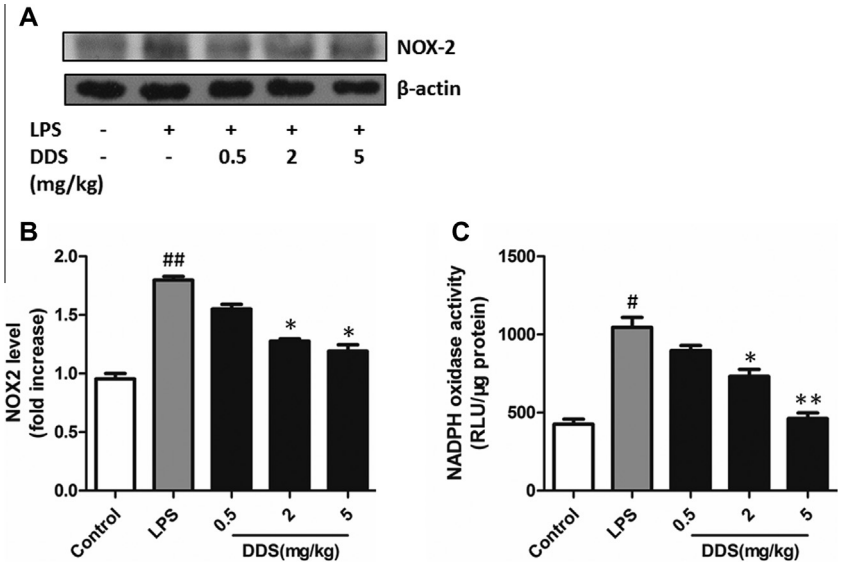

Fig. 4. LPS-induced NOX2 expression and NADPH oxidase activity were reduced by DDS. (A) C57BL/6J mice were i.p. injected with vehicle (control), 1 mg/kg LPS (LPS) or different concentrations of DDS (0.5, 2 or 5 mg/kg) 30 min after LPS administration, expression of NOX2 in brain vessels was examined with western blot ($n = 6$). β-Actin was used as loading control. Quantitative analysis of the expression levels of NOX2 (B). Activity of NADPH oxidase was assessed using lucigenin enhanced chemiluminescence ($n = 6$). Data are means ± SEM ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. control; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. LPS group).

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LPS is known to stimulate inflammatory response, activates NF-κB pathway and generates a variety of pro-inflammatory cytokines (e.g. TNF-α, IL-8, IL-1β) [27,28]. These would enhance oxidative injuries via modulating enzymes (such as NADPH oxidase) responsible for ROS generation and aggravated BBB damage [30,31]. DDS has been proved to suppress NF-κB p65 activation and production of IL-8 and TNF-α in LPS-stimulated animal and cell models [32,33], and has the effect of inhibiting neutrophil recruitment [26] and myeloperoxidase activity [16]. We here examined effect of DDS on oxidative stress status in brain vessels of LPS-challenged mice. We observed increase of ROS in brain vessels of LPS administrated mice, while DDS treatment decreased ROS level, as previous reported that DDS lowering ROS production in *C. elegans*, non-phagocytic human diploid fibroblasts and a mouse lung injury model [20–22]. An important source of ROS in BBB is NADPH oxidase, several NADPH oxidase homologs have been found in BBB, among which, down-regulating NOX2 expression can protect BBB from diverse stimuli that produce cerebrovascular dysfunction [11–13]. Our results showed that LPS increased NOX2 expression and NADPH oxidase activity in brain vessels of mice, while DDS counteracted the effect of LPS, indicated that DDS decreased ROS level may through reducing NOX2 expression and NADPH oxidase activity.

In this study, in order to elucidate whether or not DDS has a protective effect on BBB integrity, mice were systematically challenged with LPS, which would induce BBB related brain damage and mimic septic encephalopathy in clinic [34–36]. As inflammation plays a crucial role in the pathogenesis of many other CNS diseases involving BBB dysfunction [37–40], such as traumatic brain injury, Alzheimer's disease and stroke, and DDS has been reported to reduce infarction volume in ischemia [18], as well as prevent pro-inflammatory factors generation [33], this LPS challenged model can also be used to identify the role of DDS in neuroinflammation related diseases via evaluating its effect on BBB integrity. Our results showed that DDS attenuated LPS-induced BBB dysfunction through reducing oxidative stress, in consistent with its previously reported neuroprotective role [15–19]. In terms of DDS's use in clinical practice, this study suggested that DDS might also exert a therapeutical effect on BBB dysfunction derived diseases, such as septic encephalopathy and stroke.

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