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Bacterial toxins activation of abbreviated urea cycle in porcine cerebral vascular smooth muscle cells

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ABSTRACT

Nitric oxide (NO) overproduction via induction of inducible nitric oxide synthase (iNOS) is implicated in vasodilatory shock in sepsis, leading to septic encephalopathy and accelerating cerebral ischemic injury. An abbreviated urea-cycle (L-citrulline-L-arginine-NO cycle) has been demonstrated in cerebral perivascular nitrergic nerves and endothelial cells but not in normal cerebral vascular smooth muscle cell (CVSMC). This cycle indicates that argininosuccinate synthase (ASS) catalyzes L-citrulline (L-cit) conversion to form argininosuccinate (AS), and subsequent AS cleavage by argininosuccinate lyase (ASL) forms L-arginine (L-arg), the substrate for NO synthesis. The possibility that ASS enzyme in this cycle was induced in the CVSMC in sepsis was examined. Blood-vessel myography technique was used for measuring porcine isolated basilar arterial tone. NO in cultured CVSMC and in condition mediums were estimated by diaminofluorescein (DAF)-induced fluorescence and Griess reaction, respectively. Immunohistochemical and immunoblotting analyses were used to examine iNOS and ASS induction. L-cit and L-arg, which did not relax endothelium-denuded normal basilar arteries precontracted by U-46619, induced significant vasorelaxation with increased NO production in these arteries and the CVSMCs following 6-hour exposure to 20 μg/ml lipopolysaccharide (LPS) or lipoteichoic acid (LTA). Pre-treatment with pyrrolidine dithiocarbamate (PDTC) and salicylate (SAL) (NFκB inhibitors), aminoguanidine (AG, an iNOS inhibitor), and nitro-L-arg (NLA, a non-specific NOS inhibitor) blocked NO synthesis in the CVSMC and attenuated L-cit- and L-arg-induced relaxation of LPS- and LTA-treated arteries. Furthermore, immunohistochemical and immunoblotting studies demonstrated that expression of basal iNOS and ASS in the smooth muscle cell of arterial segments denuded of endothelium and the cultured CVSMCs was significantly increased following 6-hour incubation with LPS or LTA. This increased iNOS- and ASS-proteins expression in both preparations was inhibited by SAL, but was further increased by AG. These results indicate that LPS and LTA induce the L-cit-L-arg-NO cycle via induction of iNOS and ASS in the CVSMCs, accounting for massively increased NO-production and cerebral vasodilation in septic shock. Simultaneous inhibition of both pathways and NFκB-activation may be necessary to efficiently decrease or normalize NO production in the CVSMCs in this disease condition, and/or prevention and treatment of cerebral vessel-related brain dysfunctions. Our results further suggest to avoid using iNOS inhibitors alone which may cause upregulation of iNOS and ASS resulted from feedback-inhibition of iNOS activity. Accordingly, combined treatments with specific iNOS-activity inhibitor and inhibitor for iNOS genomic expression may provide a

Abbreviations: AG, aminoguanidine; AS, argininosuccinate; ASL, argininosuccinate lyase; ASS, argininosuccinate synthase; cNOS, constitutive nitric oxide synthase; CVSMC, cerebral vascular smooth muscle cell; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; IFN, interferon; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; L-arg, L-arginine, L-cit, L-citrulline; LPS, lipopolysaccharide; LTA, lipoteichoic acid; NLA, nitro-L-arginine; NO, nitric oxide; PDTC, pyrrolidine dithiocarbamate; TNF, tumor necrosis factor.

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strategy in optimally managing brain sepsis and related encephalopathy associated with enhanced iNOS expression and NO overproduction.

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1. Introduction

LPS and LTA components of bacterial cell walls of the respective gram-negative and gram-positive organisms have been shown to play a central role in the pathogenesis of septic shock. In the early stage of hypotensive septic shock, the dynamic cerebral autoregulation was improved after LPS, suggesting that the magnitude of the cerebrovascular response to a given physiological change in the mean arterial pressure (MAP) is similar, but that the cerebral circulation responds more rapidly to a given change [1]. In late stage of septic shock, however, the hyporesponsiveness of cerebral arteries together with systemic hypotension has been thought to blunt auto-regulation and results in acceleration of cerebral ischemic injury and brain dysfunction (septic encephalopathy) [2–5]. LPS and LTA stimulate the endogenous release of pro-inflammatory cytokines [interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ]] that stimulate the expression of inducible nitric oxide synthase (iNOS) in the endothelial cell (EC), vascular smooth muscle cell, and perivascular nerve [4]. Hypotension in septic shock, and subsequent septic encephalopathy and organ dysfunctions appear to involve massive production of NO in the vascular wall [4–8].

Current evidence indicates that, although intracellular L-arg levels in endothelial cells exceed the apparent Km for endothelial NOS (eNOS), most of this is unavailable for NO production under normal physiological conditions. NO production in the endothelial cell is dependent on the regeneration of L-arg from L-cit via ASS [9,10]. No high-output iNOS activity can be induced in human endothelial cells by immunostimulants such as LPS, IFN-γ, TNF-α, and IL-1 [11]. Furthermore, constitutive NOS (cNOS) activity decreases in human endothelial cells under the influence of inflammatory stimuli, and mRNA for cNOS decreases after exposure to IFN-γ and LPS without de novo expression of iNOS mRNA [11]. This raises the question as to the cellular site for primary high output NO production responsible for vasodilation and refractory shock in sepsis. Despite the recognition that iNOS induction leads to NO production, and high output NO production central to vascular dysfunction in septic shock, the exact mechanisms whereby this occurs, particularly, in the cerebral vascular smooth muscle cell are not completely understood.

According to urea cycle, L-cit in the presence of L-aspartate is converted to form argininosuccinate (AS) catalyzed by ASS enzyme. AS is then cleaved by argininosuccinate lyase (ASL) to form L-arg, the substrate for NOS, and fumarate [12]. It is notable that under physiological conditions a variety of cell types lacking a complete urea cycle often possess the urea cycle enzymes ASS and ASL which confer the ability to regenerate L-arg from the NOS product, L-cit [13–16]. This L-arg-L-cit cycle has been shown in cerebral perivascular nitrergic nerves [14,15,17,18] and endothelial cells [16,18], but not in the vascular smooth muscle in normal porcine cerebral arteries [16–18]. Morphological studies have further demonstrated that iNOS, ASS, and ASL are completely localized in the same neuronal fibers and endothelial cells [14–16], allowing efficient recycle of L-cit to form L-arg, and synthesis of NO. The lack of conversion of L-cit to L-arg in normal vascular smooth muscle cells is due to very low or lack of expression of constitutive ASS in these cells [14–16,19,20]. The ASS and iNOS enzymes, however, can be induced in vascular smooth muscle cells under certain conditions such as septic shock [19–21] and it is plausible that vasodilation is prompted by NO synthesized within the vascular smooth muscle cells.

The purpose of the present study was to delineate the mechanisms of LPS- and LTA-induced increase in NO production in cerebral arterial smooth muscle cells using in vitro blood vessel myography, immunocytochemistry, and immunoblotting methods. Our results indicate that induction of the L-cit-L-arg-NO cycle by LPS and LTA in the vascular smooth muscle cell of porcine cerebral arteries is via co-induction of iNOS and ASS.

2. Materials and methods

2.1. Cell culture

Primary culture of cerebral vascular smooth muscle cell (CVSMC) was performed according to the method described by Tao et al. [19]. Fresh heads of adult pigs (60–100 kg) of either sex were collected from local packing companies (*Excel Corporation*, *Beardstown*, *IL,* and *Hsien Meat Market Company Limited*, *Fong-lin country*, *Hualien*, *Taiwan*). After craniotomy, the entire brain with dura attached was removed and placed in ice-cold sterile phosphate-buffered Saline (PBS) (140 mM NaCl, 4 mM KCl, 1 mM, KH_2PO_4, pH 7.4) containing antibiotics (100 μg/ml penicillin G potassium, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml fungizone, *Biological industries*, *Israel*). The basilar arteries, anterior and middle cerebral arteries, and the internal carotid arteries were removed and cleaned of surrounding tissue under a dissecting microscope. The vessels were then placed on a sterile petri dish and the luminal surface was rubbed with the sterile cannula of the appropriate size for removal of endothelial cells. The vessels were then slit open longitudinally and cut into approximately 2 × 2 mm explants, placed in DMEM (Dulbecco's Modified Eagle Medium, *Hyclone Laboratories*, *USA*) containing antibiotics and washed once by centrifugation at 1000 rpm for 5 min and the explants were plated in 35 mm 6 well cultured plates in DMEM containing antibiotics plus 5% fetal bovine serum (FBS, *Hyclone Laboratories*, *USA*), and placed in an incubator in an atmosphere of 5% CO_2 in air at 37 °C. After 7–10 days when the cells had exited, the tissue was removed, and the medium changed every 4–5 days until the cells reach confluence. Cells were then passaged into 6-well culture plates (35 mm) using 0.25% trypsin (*Gibco*, *Life Technologies*, *NY*, *USA*) and grown in DMEM plus 5% FBS and antibiotics. To confirm that cells were of smooth muscle origin, each isolated batch grown to confluence was split onto a coverslip and immunolabeled with a monoclonal antibody to smooth muscle α-actin using a kit (SIH 903-A) obtained from Sigma. The smooth muscle cells were identified by their characteristic hill and valley pattern and staining for α-actin smooth muscle. Our primary cultures stained >95% positive for α-actin smooth muscle. Cells in passage 3–6 were used for immunohistochemical and immunoblot experiments.

2.2. Bio-imaging of NO production in CVSMC

The use of NO-reactive fluorescent indicators has allowed the bio imaging of nitric oxide in conjunction with fluorescence microscopy [22,23]. The cerebral vascular smooth muscle cells (CVSMCs) grown on coverslips were treated with LPS (20 μg/ml) or LTA (20 μg/ml) or untreated (control), and then incubated for 12 h at 37 °C in PBS (+)[phosphate buffered Saline (PBS) containing 10 μM diaminofluorescein (DAF) in 0.02% DMSO] for loading, and washed with PBS (pH 7.4). The coverslips were transferred to a confocal field visualizer and mounted on a confocal laser scanning microscope (Olympus fluoview) and fluorescent images were stored and analyzed for fluorescence using the scan-imaging software (fluoview) as described in immunohistochemical methods. At least 10 cells were analyzed per image captured and a

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