Lipopolysaccharide Induces Subacute Cerebral Microhemorrhages with Involvement of Nitric Oxide Synthase in Rats

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Background: Cerebral microhemorrhage (CMH) is a neuropathological term that could be easily found in cerebral amyloid angiopathy, intracerebral hemorrhages, etc. CMHs could be detected clearly in vivo by magnetic resonance imaging (MRI)–susceptibility-weighted imaging or MRI T2* scan. This terminology is now accepted in the area of neuroimaging. CMHs are quite common in elderly patients and are associated with several other neuropsychiatric disorders. The causes of CMHs are complicated, and neuroinflammation is considered as one of the well-accepted mechanical factors. This study investigated whether lipopolysaccharide (LPS)-induced CMHs occur through the regulation of nitric oxide synthase (NOS) isoforms and reveals the exact underlying mechanism of LPS-induced CMHs. *Methods:* Our work successfully developed a subacute model of CMHs in rats. LPS was intraperitoneally injected into rats at 0, 6, and 24 hours, which induced typical CMH features 7 days after the injection. These could be detected on the brain surface or parenchyma by hematoxylin and eosin staining and MRI. *Results:* LPS-treated rats showed significant activation of astrocytes and microglia, as well as loss of pericytes and disruption of blood–brain barrier. Meanwhile, both astrocytes and microglia were positively correlated with CMH numbers. Furthermore, the expressions of NOS isoforms were also examined, and the levels of neuronal NOS and endothelial NOS were found to be elevated. *Conclusions:* These results implied that the NOS isoforms might be involved in the subacute model of CMHs in rats induced by LPS. **Key Words:** Cerebral microhemorrhages—neuroinflammation—nitric oxide synthase—rats.

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Introduction

Classical cerebral microhemorrhages (CMHs) refer to tiny perivascular deposits of blood degradation products such as hemosiderin from red blood cells in the brain.[1] CMHs or small intracerebral hemorrhages (ICHs) are commonly seen in the elderly,[2] particularly in those with stroke-associated cognitive dysfunctions, such as cerebral amyloid angiopathy (CAA), cerebral infarcts, and vascular dementia.[2,3] Currently, CMH has been the focus of research, and this might be due to the accumulation of CMHs, which have been linked to cognitive as well as neuropsychiatric dysfunctions.[4,5] The prevalence of CMHs ranges between 6.5% in persons aged 45-60 and 37.5% in those aged 80 years or older, respectively,[2] and the presence of CMHs was associated with an increased risk of development of ICH.[6]

From the current point of view, there are 3 subtypes based on the small vessel wall alterations, that is, pathological changes found in CMHs: lipohyalinosis, arteriolosclerosis, and CAA.[7] Currently, the accepted animal models of CMHs are induced by Amyloid Protein Precursor (APP) transgenic,[8] hypoxia–reoxygenation, or hypertension.[9] These existing animal models have several disadvantages: (1) the presence of CMH development in these models can take up to 15-24 months, and (2) invasive surgical procedures are required to exacerbate the appearance of CMHs. Mostly importantly, clinically, CMHs may develop independent of amyloid deposition, hypoxic brain injury, or hypertension.[1] As a result, limited information is available on the pathophysiology of CMHs. Recent experimental studies have revealed that CMHs result in secondary brain damage via mechanisms similar to those identified in ICH,[10,11] such as blood–brain barrier (BBB) disruption and matrix metalloproteinase expressions.

One common feature of CMHs is systemic inflammation. In 2014, Fisher et al[12] developed a model of acute CMHs in mice using lipopolysaccharide (LPS) that is derived from *Salmonella* Typhimurium. Two years later, the same researchers managed to induce a subacute model of CMHs in a similar way.[1] LPS is considered as a standardized inflammatory stimulus, and intraperitoneal injection of LPS has been well-proven to cause inflammation. However, inflammation-induced rat models of CMHs are rarely reported, and hence, we aimed to introduce a rat model of CMHs.

Neuroimaging assists in diagnosing CMHs. CMHs are usually found after a period of time (i.e., after 3-6 months) clinically, which implicating the appearance of acute or sub-acute asymptomatic ICH till that period. Therefore, the subacute period of CMHs remained the focus of attention in our study, because several interventions could be chosen with the occurrence of "pre-Cerebral Microbleeds (CMB) pathology."

In late years, nitric oxide synthase (NOS) was found to be involved in neuroinflammation.[13] There are at least 3 isoforms of NOS, namely, neuronal nitric oxide synthase (nNOS), inducible nitric oxide synthase (iNOS), and endothelial nitric oxide synthase (eNOS). In the brain, all 3 NOS isoforms are expressed constitutively or inducibly, and are implicated in a number of physiological as well as pathophysiological functions.[14] An increasing body of evidence has demonstrated that upregulation of NOS and subsequent peroxynitrite (ONOO—) formation exert a devastating effect on the damage of BBB.[15] However, the exact way of LPS modulation of NOS is far from clear, and some results are contradictory.[16] For this reason, we need the second assumption that LPS-induced CMHs occur through the regulation NOS isoforms. The present study aimed to reveal the exact underlying mechanism of LPS-induced CMHs.

Materials and Methods

Animal Models

Male Sprague-Dawley rats, aged 10 weeks (weighting 280 ± 20g), were obtained from the Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). The housing condition was similar to previously reported work.[17] Forty rats were divided into 2 groups—LPS-treated rats (n = 20) and phosphate-buffered saline (PBS)-treated rats (n = 20). The LPS-treated rats were treated with 1 mg/kg dose of LPS derived from *Salmonella* Typhimurium (Sigma-Aldrich, St. Louis, MO) (LPS-treated rats) or 1 × PBS injection intraperitoneally injected at 0, 6, and 24 hours. The rats were then sacrificed 7 days after the first injection to examine the development of subacute CMHs. The rats were fed and drank ad lib. Seven days after the first injection, the rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (1 mL/300g). Cardiac perfusions were performed using ice-cold 1 M PBS for 5 minutes to clear the cerebral vasculature, and brains were processed for gross observation of CMHs.

Histology of Hematoxylin and Eosin (H&E) Staining

Brains were fixed in 4% paraformaldehyde at 4°C for 24 hours. The postfixed brains were cryoprotected in PBS containing 25% sucrose. The brains were then sectioned into 10 mm with a cryostat (CM1900, Leica, Shanghai, China). One section was selected from every 100 mm. For the detection of CMHs, H&E staining was performed according to the manufacturer's protocols (Solarbio, Beijing, China). Bright-field analysis was done using a Leica DM4500B fluorescence microscope (Leica, Wetzlar, Germany). Pictures were taken with Leica IM50 Image Manager Software (Leica, Cambridge, UK).

Immunofluorescence for glial fibrillary acidic protein (GFAP), Iba-1, β-Platelet-derived growth factor receptor (β-PDGFR), and Laminin

To determine the role of BBB damage and neuroinflammation in LPS-induced CMH development,