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Possible involvement of NO/NOS signaling in hippocampal amyloid-β production induced by transient focal cerebral ischemia in aged rats

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ABSTRACT

In the present study, to define the roles of nitric oxide (NO) signaling in amyloid-β (Aβ) production after transient cerebral ischemia, extracellular levels of NO and Aβ were monitored by intracerebral microdialysis in the hippocampus of aged rats exposed to transient middle cerebral artery occlusion and reperfusion (MCAO/R). The results indicated that 1-h MCAO significantly upregulated hippocampal NO and Aβ levels. In addition, the NO elevation preceded the Aβ changes. The Western blotting suggested that acute hypoperfusion could increase the expression of β-secretase 1 (BACE1) but not BACE2. The enhanced NO concentration in acute stage of MCAO/R was coincident with increased eNOS expression, while in subacute stage was coincident with increased iNOS and nNOS. Our results also indicated that pretreatment of L-NAME, one non-selective NOS inhibitor could decrease the BACE1 expression, reverse both NO and Aβ changes and rescue the delayed neuronal death. These preliminary findings indicated that activation of NOS/NO signaling system could trigger Aβ production through BACE1 pathway during acute ischemic episode. The present data may be important in understanding, at least in part, the pathological role of NO/NOS system involved in hippocampal Aβ production and neuronal damage induced by transient cerebral ischemia.

There is close association between ischemic stroke and neurodegenerative disorders such as Alzheimer's disease (AD), since more than a third of AD patients exhibit variable cerebrovascular pathology and white matter injury induced by cerebral hypoperfusion [12,17,19,31,32,45]. Furthermore, chronic brain hypoperfusion can facilitates β-secretase cleavage amyloid precursor protein (APP) cleavage and amyloid-β (Aβ) fragments deposition [3,6,38,21]. Besides chronic ischemia animal models, transient ischemic episode stimulates BACE1 expression by a mechanism requiring γ-secretase activity [2,39]. However, the roles of vascular-related risk factors, especially nitric oxide (NO) and NO synthase (NOS), underlying Aβ production have not been defined.

NO is an important messenger molecule involved in many physiological and pathological processes [26]. Published reports have shown contradictory results, with both toxic and protective effects of NOS inhibition. NOS inhibitors have been reported to reduce cerebral blood flow (CBF) and promote brain tissue damage after experimental focal cerebral ischemia [14,42]. However, there are still some research reports indicate that NOS inhibitors may have neuroprotective effects [9,11,24,28,35,37]. Selective inhibitors of nNOS have demonstrated more consistent protective results [15] suggesting that this dualism is related to the different isoforms of NOS. During ischemia reperfusion injury, NO (produced mainly by eNOS) mediates effects that would be protective following cerebral ischemia. In contrast, excessive NO (produced initially by nNOS, and later by iNOS) also mediates the neurotoxicity effect of cerebral ischemia [7,13,30,33,40].

Transient occlusion of the middle cerebral artery (MCAO) in rats leads to increased β-secretase activity and abnormal expression of Aβ peptides in brain [43]. Aβ then stimulates reactive oxygen species production, changes mitochondria activity and leads to apoptosis both in vitro and in vivo [1,20,41]. However, it is not known if NO/NOS signaling could trigger Aβ production during and after transient cerebral ischemia.

In the present study, we used well-characterized rat model of MCAO/R to mimic acute cerebral ischemia [27,34]. The objective of this preliminary study was to determine whether activated NO/NOS signaling during transient MCAO/R could initiate hippocampal Aβ production, protein marker associated with neurodegeneration and cognitive impairment.

Ten-month-old male Sprague–Dawley rats were obtained from Experimental Animal Center of Shenyang Pharmaceutical Univer-

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sity and housed under standard conditions. Rats were randomized into three groups: animals of groups 1 and 2 were exposed to 1-h MCAO; animals of groups 3 were subjected to sham occlusion. Animals of group 2 were treated with L-NAME (10 mg/kg, i.p.) 30 min before MCAO operation, while rats of group1 and 3 were treated with saline. In each experimental group, six to seven animals were studied.

The method of in vivo microdialysis used for the present study has been previously described [44]. In brief, the microdialysis probe was gently positioned in the left hippocampus corresponding to the reference of rat brain stereotaxic coordinates. Modified Reinger's lactate solution was perfused into the brain at a rate of 2 μL/min with an infusion micropump. The collected infusate was immediately transferred onto ice for measurement. The microdialysis probe was then removed from the rat under short halothane anesthesia (2%), with the guide cannula fixed on the skull. The rat was returned to a home cage and allowed to take food and water ad libitum. To monitor NO and Aβ production in the subacute stage, the microdialysis probe was reimplanted in the same rat under short anesthesia 9, 21 and 45 h after reperfusion, and microdialysis was also performed under freely moving condition. The perfused dialysates collected every 30 min from 24 to 25 h and 48–49 h afterward, were measured and averaged. Seven days after reperfusion, the correct insertion of the probe and the occurrence of delayed neuronal death were evaluated.

To measure NO level in the perfusates, the content of NO metabolites, NO_x^- (i.e., NO_2^- and NO_3^-), were determined using a HPLC-diazotization detecting method as previously described [25]. The NO_x^- level in the perfusate was converted into a percentage taking the basal level as 100%. Microdialysis samples were also analyzed for Aβ by using a denaturing, sandwich ELISA specific for human Aβ1 – *x* as described previously [5].

For MCAO/R, an intraluminal filament model was used [4,36]. The MCAO procedure leading to focal ischemia was conducted under chloral hydrate anesthesia. Rectal temperature was monitored throughout the surgical procedures and maintained normothermic (37.0 ± 0.5 °C) by a heating blanket controlled by an electronic temperature controller. To achieve a transient MCAO, the internal carotid artery (ICA) was exposed, and a 3–0 monofilament nylon suture was introduced into the ICA lumen through a puncture and was gently advanced to the distal internal carotid artery (dICA) until resistance was felt. After 1 h, the suture was withdrawn from the ICA and the dICA was immediately cauterized.

The brain protein extraction was prepared according to the methods as described previously [10]. Samples containing equivalent amounts of protein were applied to 10–15% acrylamide denaturing gels (SDS-PAGE). Proteins were then transferred to an Immobilon PVDF transfer membrane (Millipore) for 1 h at 50 V. Membranes were blocked in 20 mM Tris–HCl (pH 7.4), 150 mM NaCl and 0.1% Tween 20 (TBS-T) containing 5% fat-free milk powder for

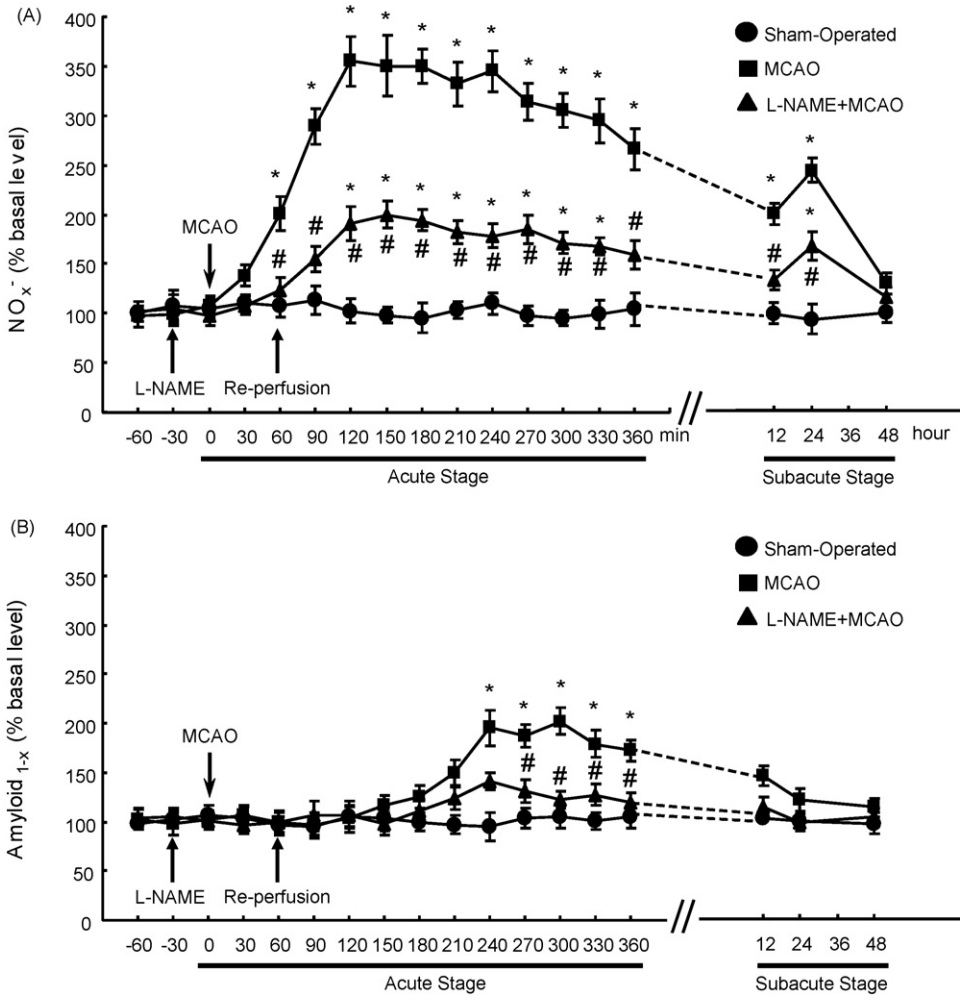

Fig. 1. L-NAME reverses the increased levels of NO_x^- and Aβ production in the hippocampus of conscious rats after 1-h MCAO. Each value represents the mean ± S.E.M. (%) of the basal level. (A) Effects of L-NAME on NO upregulation at the acute and subacute stage after MCAO/R. (B) Effects of L-NAME on Aβ production at the acute and subacute stages after MCAO/R. Vehicle or 10 mg/kg L-NAME was administered intraperitoneally 30 min before ischemia as shown by the arrow. $^*P < 0.05$ versus each corresponding sham-operated group; $\#P < 0.05$ versus each corresponding vehicle-treated ischemic group.

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1 h at room temperature and incubated with antibodies. All incubations were done overnight at 4 °C. After washing, membranes were incubated 60 min at room temperature with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody diluted in TBS-T. To quantitate the immunoreactivities, Western blots were scanned and analyzed using Scion Image software (Scion Corporation, Frederick, MD, USA).

Data in the figures represent mean ± S.E.M. All statistical analysis was performed by using SPSS 13.0 for Windows. The results of the microdialysis analysis were expressed as the percentage values of the basal level. The data were analyzed by one-way or two-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test for multiple comparisons among different groups. Differences with $P < 0.05$ were considered significant.

Before MCAO, the basal level of NO_x^- and Aβ peptide in 30-min dialysate samples from animals of three groups were not significantly different (Fig. 1). After MCAO, the levels of NO_x^- and Aβ in the sham-operated rats were still stable over a period of 360 min in acute stage (Fig. 1). In the vehicle-treated ischemic animals, the levels of NO_x^- significantly increased 60 min after the beginning of MCAO ($P < 0.05$; Fig. 1A), and the significant elevation continued over 360 min. The Aβ level in the vehicle-treated ischemic rats was also increased at 210 min ($P < 0.05$; Fig. 1B) and returned to the basal level 12 h after MCAO/R. Microdialysis data also indicated that the increased Aβ production was preceded by NO elevation.

At the subacute ischemia stage, NO_x^- level in vehicle-treated ischemic group was still higher than the basal level at 24 h ($P < 0.05$), and declined to basal level at 48 h. No Aβ level changes were detected at the subacute ischemia stage, 12, 24 or 48 h after MCAO.

Pronounced eNOS elevation was detected 2 h after the beginning of ischemia ($P < 0.01$) and returned to basal level at 24 h. A slight elevation in nNOS and iNOS was also seen at 12 and 24 h after transient MCAO and declined at 48 h (Fig. 2). BACE1 protein levels but not BACE2 were increased 4 h after MCAO (Fig. 3).

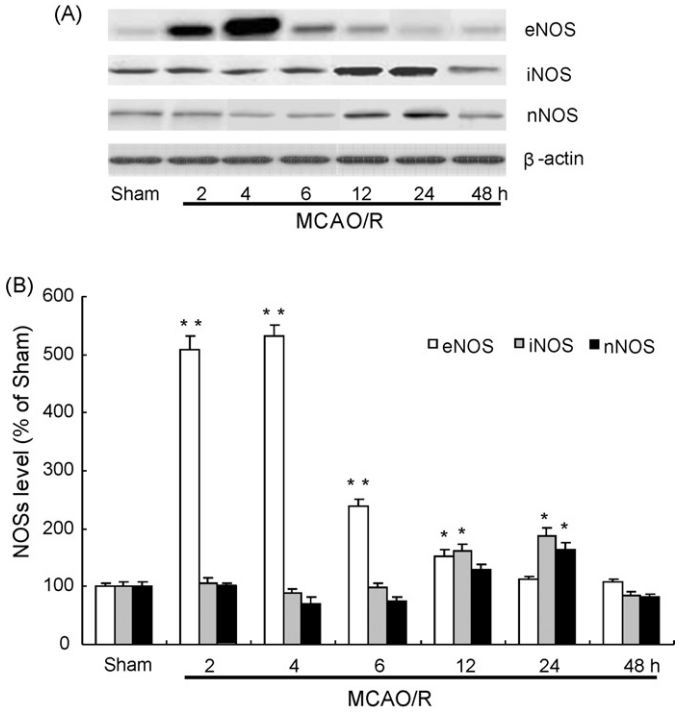

Fig. 2. MCAO/R-induced NOS isoforms changes. Proteins were separated on SDS-PAGE and analyzed by Western blotting. Representative immunoblotting image showing NOS expression following MCAO/R at indicated time points. Quantitative analysis of NOS levels was performed by densitometric analysis of immunoblots. Data are expressed as percentages of values of sham-operated animals (mean ± S.E.M.). *$P < 0.05$; **$P < 0.01$ versus sham-operated animals.

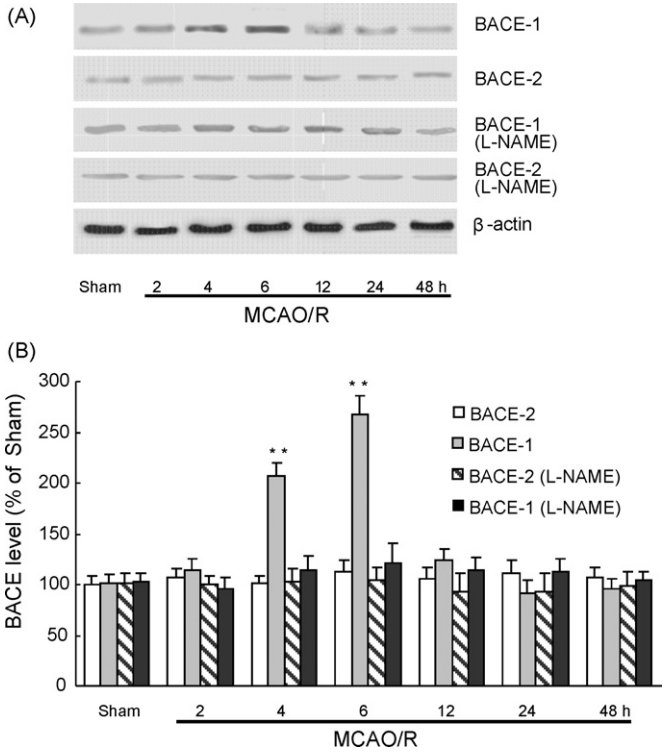

Fig. 3. L-NAME reversed MCAO/R-induced temporal changes of BACE in aged rats. Proteins were separated on SDS-PAGE and analyzed by Western blotting. Representative immunoblotting image showing BACE1 and BACE2 expression following MCAO/R at indicated time points. Quantitative analysis of protein levels was performed by densitometric analysis of immunoblots. Data are expressed as percentages of values of sham-operated animals (mean ± S.E.M.). *$P < 0.05$; **$P < 0.01$ versus sham-operated animals.

At the acute stage, pretreatment with L-NAME significantly prevented the upregulation of NO_x^- levels after MCAO/R ($P < 0.05$; Fig. 1). Furthermore, L-NAME also reversed the BACE1 overexpression (Fig. 3) and Aβ production (Fig. 1). At the subacute stage, the increase in NO_x^- levels at 24 h was still prevented by L-NAME ($P < 0.05$). The NO_x^- levels and Aβ production at 48 h were not significantly different from other two groups (Fig. 1).

Seven days after 1-h MCAO, the viable neurons in the hippocampus and hippocampal CA1 region of vehicle-treated rats were reduced ($P < 0.01$; Fig. 4C, D and G). Pretreatment with L-NAME significantly reversed the neurons damage ($P < 0.05$; Fig. 4E, F and G), whereas those remained significantly reduced compared with sham-operated animals ($P < 0.05$; Fig. 4A, B and G).

NO/NOS signaling pathway and beta/gamma secretase have been previously well documented to be involved during permanent or transient cerebral ischemic episode [2,3,6,21,38,39]. However, the detail interactions between these molecules were still far from clear demonstration. The findings from present study through microdialysis indicated that a biphasic upregulation of hippocampal NO levels occurred 2 and 24 h after MCAO/R. The increased eNOS expression was responsible for the first NO upregulation, while nNOS and iNOS for the second. Moreover, the microdialysis results indicated that Aβ production was also enhanced significantly 4 h after MCAO and was preceded by eNOS overactivation and the first NO elevation.

The first NO production observed during the first 2 h after MCAO might be a response to several reasons: (1) the change in shear stress caused by MCAO. Nitric oxide overproduction can be induced by eNOS upregulation in response to increased shear stress during early stage of ischemia [6,18,22,40]. (2) The changes in neurotoxins caused by MCAO. The burst of NO production may also be the

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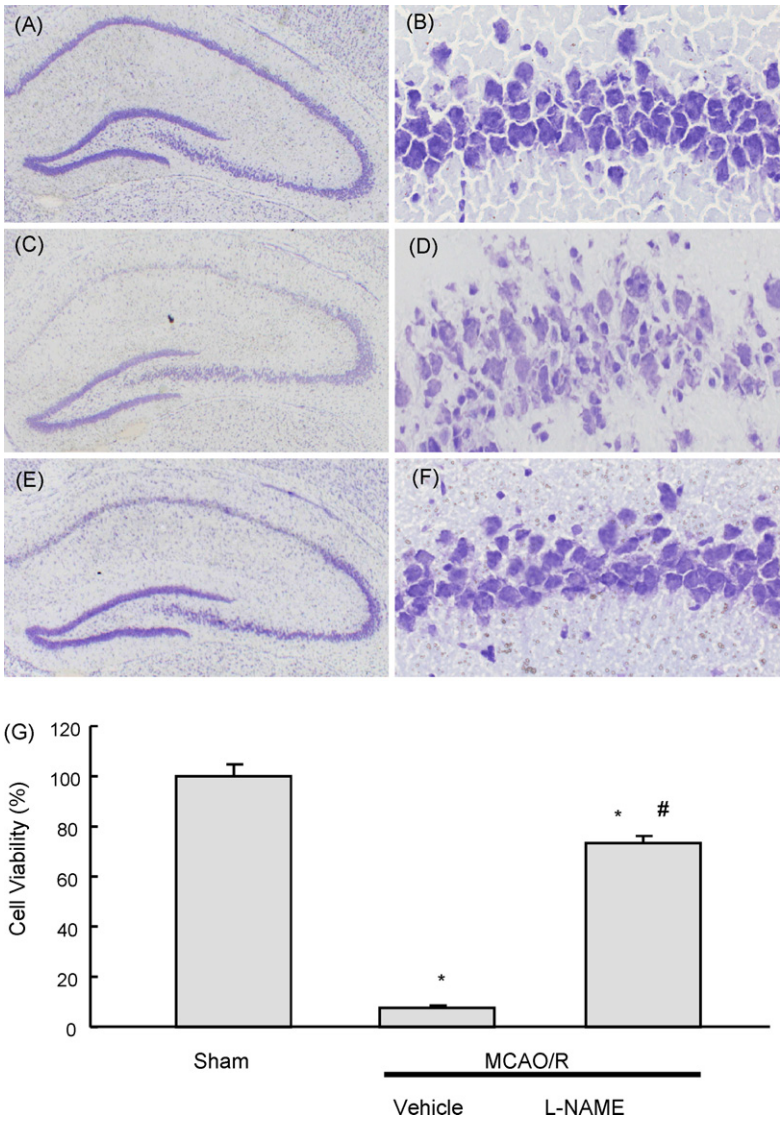

Fig. 4. Delayed neuronal death after 1-h MCAO/R is reversed by L-NAME. Representative histological sections of hippocampus (A, C, and E) and the hippocampal CA1 region (B, D, and F) in sham-operated (A and B), vehicle-treated ischemic (C and D), or L-NAME treated ischemic rats (E and F) are shown. Cell viability was expressed as percentages of the averaged number of viable cells from sham-operated rats (G). Data represent the mean ± S.E.M. $^{*}P < 0.01$ versus sham-operated; $\#P < 0.01$ versus vehicle-treated ischemic rats.

result of increased glutamate or Aβ release during ischemia and be responsible for the neuronal damage [8,29].

BACE1 and BACE2, define a family of transmembrane aspartic proteases. BACE1 exhibits all the properties of the β-secretase, and is the key enzyme that initiates the generation of Aβ. BACE1 is principally present in the central nervous system, while BACE2 is not highly expressed in the brain. Our observation indicated that transient cerebral ischemia only caused significant increase in BACE1 protein level, but not BACE2. These results were consistent with previous study [43].

The second NO peak might be due to the increased iNOS/nNOS expression and Aβ production during subacute stage of MCAO/R. It has been reported that nNOS activity increases 10 min after focal ischemia and returns to the basal level 60 min later [16,18]. However, in our study, nNOS expression was upregulated only during the later phase of MCAO/R (12–24 h after ischemia) together with the iNOS isoform. The possible reason for such a dynamic changes of iNOS/nNOS might be due to the elevation of Aβ level. Previous research reports have suggested that injection of Aβ causes an increase of nNOS and iNOS immunoreactivity in the temporal cortex and hippocampus, and the enhanced NO level mediated the neurotoxicity of Aβ [1,23].

Increasing evidences have shown that NOS/NO mediates the downstream signal transduction of various neurotoxins (e.g. Aβ) leading neuronal injuries. In primary cultured neurons, NO mediates the neurotoxicity induced by Aβ and in animal models, the inhibition of NO generation can reduce the infarct size of the brain caused by MCAO in rats. More importantly, abnormal overactivation of NOS and subsequently excessive production of NO mediates neuronal cell death. Previous in vitro studies have reported that NOS inhibitor (L-NAME) or NO scavengers (carboxy-PTIO and hemoglobin) significantly reduce cell death induced by Aβ, L-arginine and glutamate in cortical neurons, supporting that NO is involved in neurotoxicity. Therefore, compounds that inhibit the activity of NOS could reverse the Aβ production and may have neuroprotective effects after ischemic injury. The results of our present research further indicated that L-NAME, one NOS inhibitor could prevent over-expression of BACE1, reversed the changes of NO/Aβ and ameliorated the neuronal damage induced by acute MCAO/R.

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In conclusion, our present study indicated that activation of NOS/NO signaling system could trigger Aβ production through BACE1 pathway during and after acute focal cerebral ischemia in aged rats. Non-selective NOS inhibitor L-NAME reversed these pathological changes. These findings suggested that NO/NOS play a critical role in cerebral Aβ production under some pathological situations.

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