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Inhibitory effects of chiral 3-*n*-butylphthalide on inflammation following focal ischemic brain injury in rats[1]

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KEY WORDS cerebral ischemia；inflammation；3-*n*-butylphthalide；neutrophils；intercellular adhesion molecule-1；tumor necrosis factor；Western blotting；*in situ* hybridization

ABSTRACT

AIM：To evaluate the degree of neutrophil infiltration into ischemic tissue after transient focal cerebral ischemia，and to examine the effects of chiral 3-*n*-butylphthalide (NBP) on this inflammatory process. **METHODS**：After a 24-h reperfusion following transient cerebral ischemia，two different techniques，histologic analysis and modified myeloperoxidase (MPO)-quantification method，were utilized to identify the infiltration of neutrophils into cerebral tissue following ischemia. The expression of intercellular adhesion molecule-1 (ICAM-1) and tumor necrosis factor-α(TNF-α) in the ischemic zone were observed by immunohistochemistry，Western blot，and *in situ* hybridization techniques. **RESULTS**：In cerebral cortex area perfused by middle cerebral artery (MCA)，MPO activity was greatly increased after 24 h of reperfusion in the vehicle group，and it correlated well with the infiltration of neutrophils. Administration of *dl*-，*d*-，and *l*-NBP (20 mg·kg^{-1}) partially inhibited both the increase in MPO activity and the appearance of neutrophils in ischemia-reperfusion sites. Up-regulation of ICAM-1 was also observed on the microvessel endothelium in the ischemic territory. In addition，chiral NBP markedly blunted ICAM-1 expression，and decreased the number of TNF-α blue purple-positive neurons induced by ischemia-reperfusion injury. **CONCLUSION**：The results indicate that the increase in neutrophils infiltration into the infarct site implicated postischemic brain injury，and NBP was effective in protecting the ischemic sites following ischemic insult.

INTRODUCTION

There is now considerable evidence indicating that an acute inflammatory response occurs after cerebral ischemia，characterized by a progressive increase in leukocyte adherence and infiltration over the initial hours upto days after the insult[1]. In the brain，circulating inflammatory cells adhere selectively to vascular endothelium adjacent to focal ischemic sites and then exhibit diapedesis. These inflammatory cells could be important contributors to brain damage caused by vascular dysfunction，endothelial cell injury，and blood-brain barrier (BBB) breakdown after stroke.

However，the precise mechanism of the neutrophils' adherence under the ischemia-reperfusion condition is still poorly understood. Recent reports have indicated that the expression of intercellular adhesion molecule-1 (ICAM-1)，which is mainly involved in leukocytes adhesion and migration steps，is up-regulated in ischemic brain tissue，and ischemia-reperfusion treatment increases CD11/CD18-ICAM-1-dependent neutrophil adherence to endothelial cells[2,3].

TNF-α is a pleiotropic cytokine with a diverse range of biological functions，most notably in immune and inflammatory reactions. It appears to be involved in inflammatory，thrombgenic，BBB，and vascular changes associated with brain injury. There are many evidences demonstrating that increased neuronal expression of TNF-α mRNA in the rat ischemic cortex precedes the leukocyte infiltration that occurs after focal stroke. In addition，TNF-α is capable of up-regulating the basal level of ICAM-1 expression on endothelial surface，which may be relevant to inflammation in CNS[4,5].

The purpose of the present series of experiments was to evaluate the protective effect of *dl*-，*d*-，and *l*-NBP on post-ischemic inflammation，especially on neutrophils

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infiltration and the expression of ICAM-1 and TNF-α in infarct zone after transient cerebral focal ischemia was produced by middle cerebral artery occlusion (MCAO) in the rat.

MATERIAL AND METHODS

Animal preparation Transient focal brain ischemia in the area perfused by MCA was induced as reported previously[6]. Briefly, adult male Wistar rats weighing 250 – 270 g, Grade Ⅱ, certificate № 01-3008, were anesthetized with chloralhydrate (0.3 g·kg^{-1}). After median incision of the neck skin, the left external carotid artery (ECA) was carefully dissected. An 18 mm length of nylon suture (φ 0.28 mm) was introduced into the transected lumen of the external carotid artery and gently advanced into the internal carotid artery (ICA) to block the origin of the left MCA. For reperfusion, the suture was withdrawn back into the external carotid artery to restore ICA-MCA blood flow. The control rats underwent surgery but did not have the suture inserted.

Neurological Testing Twenty-four hours after surgery, each animal was graded groded by using the neurological grade (grading of 0 to 3) to define the degree of contralateral forelimb paralysis resulting from the focal ischemic damage in ipsilateral cortex[7].

Histology Following the neurological evaluation, animals were anesthetized and perfused transcardially with 100 mL of physiological saline (25 ℃) to flush all blood components from the vasculature. Thereafter, the brain samples of the ischemic hemispheres were taken from the skull and postfixed overnight in paraformaldehyde. After embedding in paraffin by routine histological procedures, 7-μm coronal sections were cut on an American Optical Reichert model 820 microtome (Reichert Scientific Instrument, USA) and stained with hematoxylin and eosin. The profile and degree of neutrophils infiltration into the brain was determined and photographed under light microscopy.

Chemical Analysis The forebrain segments were immediately frozen in liquid nitrogen for later use. The method used to quantify MPO activity from rat brain samples was similar to that described by Barone *et al*[7,8], but with minor modifications. The modified MPO assay for brain tissue was conducted as follows. The animals were sacrificed and perfused transcardially with 100 mL isotonic saline (25 ℃) immediately. Brain samples from the infarcted areas were thawed on ice and wet weight in grams was measured. Each sample was homogenized (1∶20 wt/vol) in potassium phosphate buffer 5 mmol·L^{-1} (pH 6.0, 4 ℃) using a polytron (Switzerland) for three on/off cycles at 5-s intervals and centrifuged at 3000 × *g* (30 min, 4 ℃). The supernatant was discarded and the pellet was washed again as described above. After decanting the supernatant, the pellet was extracted by suspension in 5 % hexadecyltrimethylammonium bromide (HTAB, Sigma Chemical Company) in potassium phosphate buffer 50 mmol·L^{-1} (pH 6.0, 25 ℃) for approximately 2 min at an original tissue wet weight volume ratio of 1∶10. The samples were immediately subjected to three freeze/thaw cycles with sonications (10 s, 25 ℃) between cycles. After the last sonication, the samples were incubated at 4 ℃ for 20 min and centrifuged at 12 500 × *g* (15 min, 4 ℃). Supernatant 0.1 mL was mixed with potassium phosphate buffer (50 mmol·L^{-1}, pH 6.0) 2.9 mL containing *O*-dianisidine dihydrochloride 0.167 g·L^{-1} (Sigma Chemical Company) and hydrogen peroxide 0.0005 %. The change in absorbance at 460 nm was measured with a spectrophotometer. One unit of MPO activity is defined as that which degrades 1 μmol of peroxide per min at 25 ℃.

Immunohistochemistry Immunohistochemical staining for the ICAM-1 antibody was performed as previously described[9]. Coronal brain section (20 μm-thick) were cut on a cryostat (SLEE Medical Equipment Ltd, UK) and thaw-mounted onto 3-amino-propyltriethoxy saline coated slides. Cryostated sections were air-dried for 1 h at 25 ℃, followed by fixation in acetone for 10 min at 4 ℃ and rinsing in PBS (pH 7.4). Sections were incubated with a polyclonal anti-rat ICAM-1 antibody (Santa Cruz Biotechnology Inc, USA) at 2 mg·L^{-1} for 30 min at 37 ℃, and biotinylated horse anti-rabbit immunoglobulin G for 30 min. The sections were sequentially incubated with streptavidin horse-radish peroxidase complex for 30 min. Antibody-binding was detected using an ABC kit according to the manufacturer's instructions and with 3,3′-diaminobenzidine (DAB) as the chromogen. Deletion of the primary antibody served as the negative control. The peroxidase-stained microvessels in the ipsilateral and contralateral hemispheres were observed under the light microscope at 200 × magnification.

Western blot analysis Rats were decapitated after 24 h of reperfusion following 1 h of ischemia, and the forebrains were removed. These samples were homogenized and centrifuged at 20 000 × *g* for 20 min at 4 ℃. After heating at 95 ℃ for 5 min, the samples were cen-

trifuged for 5 min at 12 000 × *g* at 25 ℃. An aliquot of the supernatant was removed for protein concentration determination. The supernatant was used as a protein sample for Western blot. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) was performed based on the primary method by Suzuki[10]. In brief, protein samples were boiled at 100 ℃ in 2.5 % SDS and 5 % mercaptoethanol, and were electrophoresised with protein molecular weight markers (Beijing Jing-ke Biochemical Reagent Company). The protein were then transferred to a polyvinylidene fluoride membrane with the transfer buffer of Tris-HCl 25 mmol·L^{-1} (pH 7.5), glycine 192 mmol·L^{-1}, and methanol 20 %. After blocking in 5 % milk, primary antibody was applied, followed by horseradish peroxidase-conjugated secondary antibody. The membranes were then developed with 3,3′-diaminobenzidine tetrahydrochloride in the presence of H_2O_2.

***In situ* hybridization** Rats used for *in situ* hybridization were decapitated after 24 h of reperfusion following 1 h of ischemia. The sections (20 μm) were cut on a cryostat at −20 ℃, collected on slides, and processed for *in situ* hybridization. The rat TNF-α cDNA probe was obtained from Beijing Medical University. Prior to hybridization, the brain sections were acetylated for 10 min, then immersed in 1.2 % triethanolamine/water followed by dropwise addition of 750 μL acetic anhydride over the sections and washed three times with phosphate buffered saline. Prehybridization was performed with hybridization buffer for 6 h at 25 ℃ in a humidified chamber followed by hybridization at 42 ℃ overnight using a hybridization solution consisting of the hybridization buffer and the labeled cDNA probe. After hybridization, the section were washed at 64 ℃ for 30 min, followed by rinsing at 25 ℃. The detection of digoxigenin-labeled hybridization products was performed by enzyme-liked immunoassay using anti-digoxigenin alkaline phosphatase conjugate (Boehringer-Mannheim, Germany). The alkaline phosphatase-catalyzed reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salt producing an insoluble blue precipitate, was used to visualize the hybridized molecules.

Statistical Analysis Each experiment group consisted of more than 4 animals. All values were presented as $\bar{x} \pm s$. To determine the statistical significance of the observed differences, the dependent *t* test was used.

RESULTS

Neurological Deficits The result of neurological testing indicated that MCAO produced a contralateral forepaw hemiparalysis and muscle weakness compared to sham-operated animal. *dl*-, *d*-, and *l*-NBP 20 mg·kg^{-1} inhibited the neurological deficit greatly, while NBP 10 mg·kg^{-1} had no effects.

Histology Many neutrophils were identified as isolated cells or as aggregates within blood vessels, attached to vessel walls, and at various stages of diapedesis from the vessels into the infarcted region, while neutrophils were observed in the sham-operated animals only infrequently. There was a marked increase in neutrophils infiltration after 24 h of reperfusion following 1 h MCA occlusion ($P < 0.01$ compared with sham group). Administration with *dl*-, *d*-, and *l*-NBP reduced the number of neutrophils (22 ± 5, 31 ± 8, 20 ± 3 *vs* 58 ± 16, respectively; Fig 1).

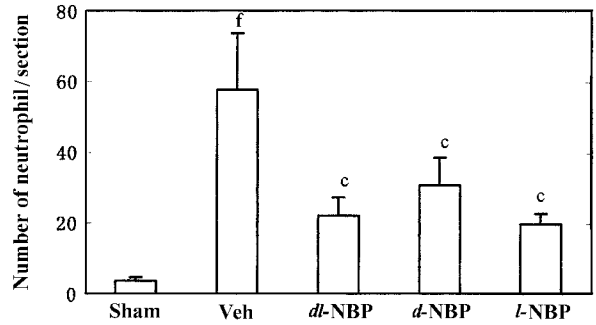

Fig 1. Effects of *dl*-, *d*-, and *l*-NBP (20 mg·kg^{-1}) on number of neutrophils in the ischemic hemisphere after 24 h of reperfusion following 1 h of MCA occlusion. The results were expressed as number of neutrophils per 5-μm section. $n = 4$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* vehicle group. $^fP < 0.01$ *vs* sham control group.

MPO Activity After 24-h reperfusion after 1-h MCAO, MPO activity was greatly increased over the segments of control group (3.6 ± 0.5 *vs* 0.37 ± 0.28 U·kg^{-1}, $P < 0.01$). *dl*-, *d*-, and *l*-NBP reduced the increase in MPO activity induced by MCAO in a dose-dependent manner (Fig 2).

Immunohistochemistry Immunoreactivity for ICAM-1 was scarcely present in sections of the sham control brains. In brain of 1 h of transient MCAO followed by reperfusion, ICAM-1 immunoreactivity was enhanced in the microvascular vessels, and vascular endothelial cells positive for ICAM-1 were ubiquitously distributed inside MCA territory but were not present on the contralateral side. The expression was greatly reduced by *dl*-, *d*-, and *l*-NBP (Fig 3).

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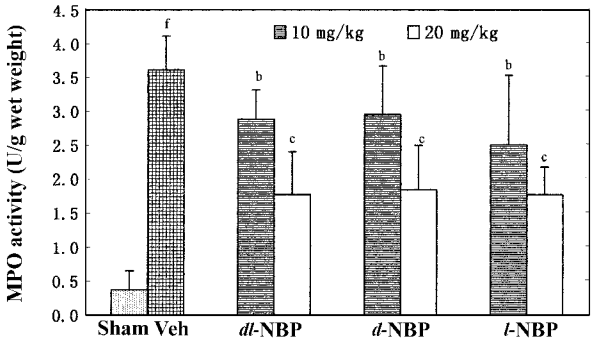

Fig 2. Effects of *dl*-, *d*-, and *l*-NBP 20 mg/kg on increased brain myeloperoxidase (MPO) activity after 24 h of reperfusion following 1 h of MCA occlusion. $n = 4$. $\bar{x} \pm s$. $^{b}P < 0.05$, $^{c}P < 0.01$ *vs* vehicle group. $^{f}P < 0.01$ *vs* sham control group.

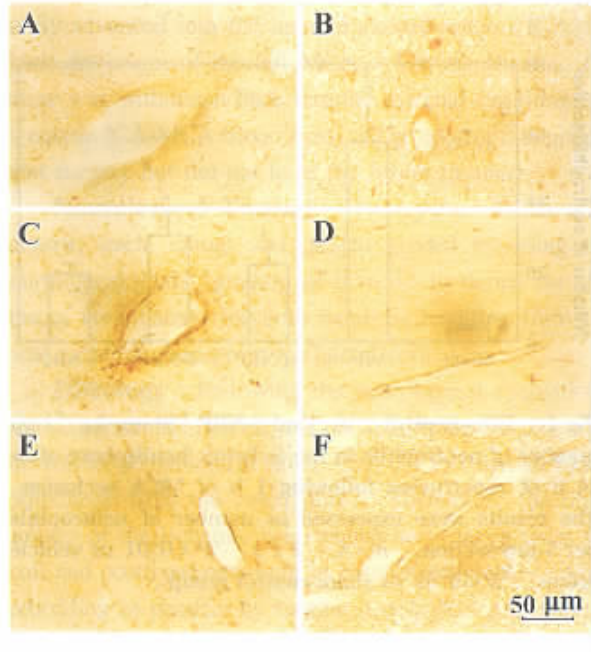

Fig 3. Effects of NBP on the changes in immunohistochemical staining against ICAM-1 in microvascular endothelial cells of the cerebral cortex after 24 h reperfusion following 1 h of transient MCAO. *dl*-NBP, *d*-NBP, and *l*-NBP were administrated 10 min and 60 min after MCAO. A: ICAM-1 antibody negative control; B: Sham; C: Vehicle; D: *dl*-NBP; E: *d*-NBP; F: *l*-NBP. ×200.

Western Blot CAM-1 protein was dramatically induced in ischemic cortex after transient MCAO in the vehicle group as indicated by Western blots, whereas no significant expression of ICAM-1 in the control group was observed. This increase in ICAM-1 protein content was markedly diminished in *dl*-, *d*-, and *l*-NBP treated groups respectively.

***In situ* Hybridization** There was no TNF-α mRNA induction in the cortex of rats in sham operated control group, cohereas TNF-α mRNA hybridization signal was significantly induced by ischemia-reperfusion injury in vehicle group, and predominantly expressed in the infarct zone. Moreover, this high TNF-α mRNA signal was blunted by administration of *dl*-, *d*-, and *l*-NBP ($20\ mg \cdot kg^{-1}$) (Fig 4).

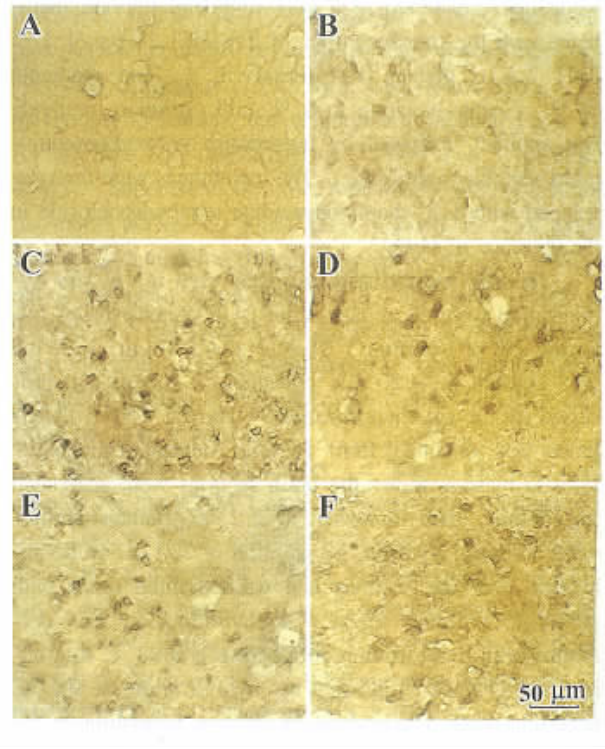

Fig 4. TNFα mRNA expression in cerebral cortex with *in situ* hybridization in MCAO rats. Histologic sections from cerebral cortex of rats were hybridized with digoxigenin-labeled cDNA probes. *dl*-, *d*-, and *l*-NBP 20 $mg \cdot kg^{-1}$ were administrated (ip) 10 and 60 min after MCAO. A: negative control; B: Sham; C: Vehicle; D: *dl*-NBP; E: *d*-NBP; F: *l*-NBP. ×200.

DISCUSSION

Post-ischemic cerebral inflammation most likely plays a critical role in ischemic brain damage. Neutrophils and monocytes are directly involved in the pathogenesis and development of ischemia-reperfusion injury by reducing microvascular blood flow, initiating thrombosis, and releasing chemical mediators such as free oxygen radicals[8]. The degree of inflammatory cell infiltration in

inflamed tissue following ischemic insult has been estimated by histological methods, or quantified by using an assay for myeloperoxidase (MPO), an enzyme for inflammatory cells (primarily neutrophils-derived)[7]. Our present study demonstrates that increased MPO activity is associated with increased neutrophils infiltration in cerebral focal ischemic tissue after transient MCAO. *dl*-, *d*-, and *l*-NBP could blunt not only the increase in MPO activity and the number of neutrophils infiltrated, but also the neurological deficit induced by MCAO. This finding suggests that NBP can reduce cerebral ischemic damage by inhibiting the post-ischemic inflammatory process.

Meanwhile, these data also show that the expression of ICAM-1 and TNF-α in the infarct site was up-regulated by transient MCAO. ICAM-1 has been implicated in neutrophil-endothelial interactions and transendothelial migration. The expression of adhesion molecules may facilitate the migration of inflammatory cells and contribute to the disease process. ICAM-1 is expressed constitutively in astrocytes and endothelia, which, however, is markedly up-regulated when the cells are treated with cytokines such as TNF-α[4]. More and more evidences have demonstrated that TNF-α activates the endothelium for neutrophils adherence and procoagulant processes that can exacerbate ischemic damage. This cytokine can enhance adhesion of inflammatory cells via increased expression of adhesion molecules, and it is possible that TNF-α may play the pivotal role in brain tissue's response to ischemia by promoting inflammatory cells infiltration. So, blocking of the TNF-α expression might be beneficial in transient ischemia[11,12].

In our present study, *dl*-, *d*-, and *l*-NBP were found to reduce the expression of ICAM-1 and TNF-α, which suggests that NBP can suppress neutrophils infiltration following ischemic insult by reducing adhesion molecules and cytokine expression. Definitive proof that NBP can decrease the expression of ICAM-1 and TNF-α directly will require more experimentation, especially by using the transgenic techniques.

We have recently demonstrated that *dl*-, *d*-, and *l*-NBP are effective in brain protection by preventing BBB damage, improving cerebral microcirculation, and increasing regional cerebral blood flow after cerebral ischemia[13-15]. In this experiment, *dl*-, *d*-, and *l*-NBP were found to attenuate post-ischemic inflammation, which might contribute to prevent the BBB damage and the dysfunction of microcirculation. Further experiments are required to clarify these issues.

In conclusion, the synthetic chiral NBP are effective in preventing the process of inflammation following ischemic insult.

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手性丁基苯酞对大鼠局灶性脑缺血后炎症的抑制作用[1]

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关键词 脑缺血；炎症；3-*n*-丁基苯酞；中性白细胞；细胞间粘附分子-1；肿瘤坏死因子；蛋白质印迹；原位杂交

目的：观察脑缺血后中性粒细胞浸润与脑损伤的关系，并研究了消旋和光活丁基苯酞对脑缺血后炎症损伤的影响. 方法：利用组织化学及髓过氧化物酶定量测定法，观察了中性粒细胞浸润程度. 同时，通过免疫组织化学方法及 Western blot，观察了脑缺血再灌后，缺血区细胞间粘附分子-1 的表达；并用原位杂交技术研究了缺血区肿瘤坏死因子（TNF-α）mRNA 表达的变化. 结果：*dl*-，*d*-和 *l*-NBP 均能明显降低缺血再灌注时脑损伤区的中性粒细胞数目及 MPO 酶的活性，并可以抑制缺血区 ICAM-1 及 TNF-α 表达的升高. 结论：中性粒细胞浸润是局灶性脑缺血后损伤的重要原因之一，NBP 可以通过抑制脑缺血后炎症的发生而产生脑保护作用.

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