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The effects of Tanshinone IIA on blood–brain barrier and brain edema after transient middle cerebral artery occlusion in rats

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ABSTRACT

Disruption of blood–brain barrier (BBB) and edema formation play a key role in the development of neurological dysfunction after cerebral ischemia. In this study, the effects of Tanshinone IIA (Tan IIA), one of the active ingredients of Salvia miltiorrhiza root, on the BBB and brain edema after transient middle cerebral artery occlusion in rats were examined. Our study demonstrated that Tan IIA reduced brain infarct area, water content in the ischemic hemisphere. Furthermore, Tan IIA significantly decreased BBB permeability to Evans blue, suppressed the expression of intercellular adhesion molecule-1 (ICAM-1), matrix metalloproteinase-9 (MMP-9), inhibited the degradation of tight junction proteins zonula occludens-1 (ZO-1) and Occludin. These results demonstrated that Tan IIA was effective for attenuating the extent of brain edema formation in response to ischemia injury in rats, partly by Tan IIA's protective effect on the BBB. Our results may have implications in the treatment of brain edema in cerebral ischemia.

Introduction

Brain edema has a crucial impact on morbidity and mortality after cerebral ischemic injury as it aggravates already impaired compliance and contributes to additional ischemic injuries due to reduced perfusion and oxygenation. Vasogenic edema, major type of brain edema, is characterized by the structural and functional impairment of blood–brain barrier (BBB) which serves as the anatomical barrier between the blood and brain parenchyma (Ballabh et al. 2004; Abbott 2000). The relative preservation of BBB integrity would be expected to minimize vasogenic edema formation in response to ischemia. Thus, drugs targeted to BBB may be a promising management strategy for treatment of cerebral ischemia.

Danshen, derived from the dried root of Salvia miltiorrhiza Bge (SM), has been widely used for treatment of cardiovascular and cerebrovascular diseases (Zhou et al. 2005; Han et al. 2008). Tanshinone IIA (Tan) is the major active ingredient of Salvia, has been shown to exert antioxidant and anti-inflammatory effect in the prevention of ischemia injury models and treatment of cerebrovascular diseases. It has a strong inhibitory effect on the inflammatory responses of rats with myocardial infarction (Ren et al. 2009). It possess neuroprotective effects on both permanent and transient focal cerebral ischemia in mice (Lam et al. 2003; Dong et al. 2009). Tan IIA can theoretically affect many aspects of the mediator cascade that can cause a permeability defect in the BBB. As so far, however, the relationship between cerebral edema, especially the BBB permeability and Tan IIA has not been well established. Accordingly, the present study was designed to explore effect of Tan IIA on BBB and cerebral edema after experimental stoke, furthermore, we studied Tan's influence on expression of the expression of intercellular adhesion molecule-1 (ICAM-1), matrix metalloproteinase-9 (MMP-9), the degradation of tight junction proteins zonula occludens-1 (ZO-1) and Occludin to further understand its protective mechanism.

Materials and methods

Reagents

Tan IIA was isolated from the roots of SM, which is based on the method described previously (Kim et al. 2004). Briefly, the methylene chloride fraction of methanol extract of SM was subjected to column chromatography over a silica gel and recrystallization. The purity of Tan IIA used was more than 99%, which was proven by high performance liquid chromatography (HPLC) according to the method for assay of Tan IIA in Chinese Pharmacopoeia. Its structure is presented in Fig. 1.

Mouse monoclonal anti-ICAM-1, MMP-9, ZO-1, Occludin were purchased from Santa Cru Biotechnology (Santa Cruz, CA). Other

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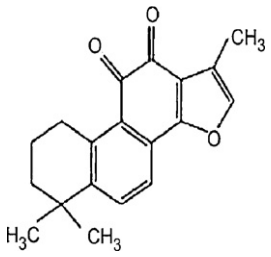

Fig. 1. Chemical structure of Tan IIA.

reagents and chemicals were purchased from Gibco (Grand Island, NY).

Animals and experimental groups

All the experimental procedures were approved by the Committee of Animal Use and Care of General Hospital of Shen yang Military Region. Sprague–Dawley (SD) rats weighing 250 ± 20 g, were randomly divided into five groups: Sham-operated group (vehicle-treated group; Tan IIA-treated group) Tan IIA was dissolved in dimethyl sulfoxide (DMSO), and was administered with different concentrations (10 mg kg^{-1}, 20 mg kg^{-1}, 30 mg kg^{-1}, respectively) through intraperitoneal injection respectively 5 min after surgery. An equivalent volume of vehicle was administered in the vehicle group.

Focal ischemia

Transient focal cerebral ischemia was induced by middle cerebral artery occlusion (MCAO) as described previously (Matsuo et al. 1994). Briefly, rats were anesthetized and maintained with a gas mixture of 98.5% air and 1.5% halothane. Right common carotid artery (ECA) was exposed through a median incision in the neck, and a 4-0 nylon suture was inserted from the ECA to the right internal carotid artery to occlude the origin of the right middle cerebral artery. After 2 h of MCAO, the suture was removed to allow reperfusion of the ischemic area via the right common carotid artery. The middle cerebral artery was not occluded after the neck incision in the sham-operated rats.

Infarct size

Rats were anesthetized and perfused with physiological saline containing 0.2% heparin 24 h after MCAO. Brains were removed, and cut into coronal sections, and immersed in a 2% 2,3,5-tripenyltetrazolium chloride solution (in phosphate-buffered saline). The infarct area (non-2,3,5-tripenyltetrazolium chloride staining area) and total brain section area were placed on transparent sheets and quantitated by Scion imaging software (Scion Corp. Frederick, MD). The percentage of the infarct area with respect to total area was calculated.

Water content

The rats were decapitated under halothane anesthesia, the brain was removed rapidly and dissected 24 h after MCAO, tissue samples from the ischemic hemisphere were weighed immediately on preweighed aluminum foil to determine the yield of wet tissue. After the tissues were dried in a desiccating oven at 110 °C for 24 h, they were weighed again to analyze the water content. Water content was calculated as (wet weight − dry weight)/wet weight × 100%.

Assessment of BBB permeability

The BBB permeability was assessed with Evans blue dye (EB) extravasation according to the method of Uyama (Uyama et al. 1988). Briefly, rats were anesthetized and EB solution (3 ml/kg, 2%) was administered intravenously 3 h before rats were killed. After a thoracotomy under anesthesia, the rats were then perfused with saline perfusion through the left ventricle to remove intravascular EB dye. The brain was removed and dissected, fresh tissue samples were weighed and homogenized in 4 ml of 50% trichloroacetic acid. The samples were then cooled and centrifuged for 30 min at 1000 rpm. The absorbance of the supernatants for EB dye was measured at 610 nm with a spectrophotometer. EB dye content is expressed as μg/g of brain tissue.

Western blotting

Brain homogenates were lysed in ice-cold lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% SDS, 0.25% NaTDC, 1 mM PMSF, 1 mg/l aprotinin, 1 mg/ml leupeptin, 1 mM DTT and 1%Triton100). The lysates were then separated by 10% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred onto polyvinylidene fluoride membrane (Millipore, MA, USA). The membranes were blocked with 5% non-fat milk powder in Tris-buffered saline (TBS) containing Tween20 (TBST) for 2 h at room temperature. Next, the membrane was incubated with antibodies against β-actin, ICAM-1, MMP-9, ZO-1 and Occludin in TBS/T containing 5% non-fat milk overnight at 4 °C. Protein expression was detected with ECL Western blotting detection reagent.

Statistical analyses

Data were expressed as mean ± SD. The statistical differences among groups were evaluated using variance (ANOVA) with Fisher's PLSD test. $P < 0.05$ was considered significant. Results were evaluated using analysis of variance and correlated by SPSS13.0 software.

Results

Tan IIA reduced infarct size and cerebral water content in the ischemic hemisphere

As seen in Fig. 2, the water content in the vehicle group was found to be significantly higher than the sham-operated group in the ischemic hemisphere. The treatment of Tan IIA sig-

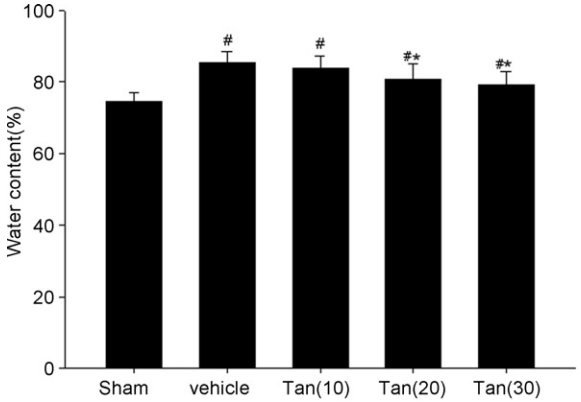

Fig. 2. Effects of Tan IIA on water content at 24 h after MCAO. Rats were pretreated with indicated concentrations of Tan IIA (mg kg^{-1}) after MCAO, $n = 6$, values are shown as mean ± SD. $^{*}P < 0.05$ *vs* vehicle group, $^{\#}P < 0.05$ *vs* sham group.

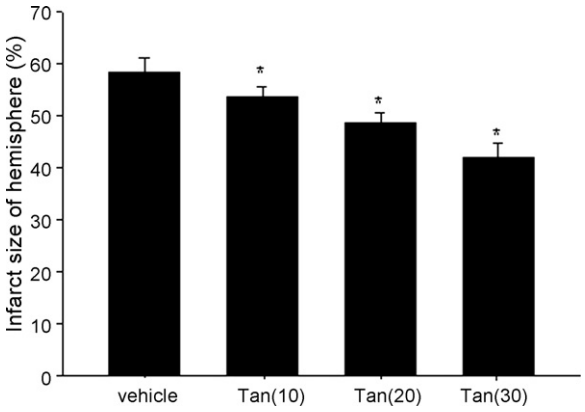

Fig. 3. Effects of Tan IIA on the infarct area. Rats were pretreated with indicated concentrations of Tan IIA (mg kg^{-1}) after MCAO. Values are shown as mean ± SD, $n = 6$, $^{*}P < 0.05$ *vs* vehicle group.

nificantly attenuated the brain edema formation as well as the infarct area determined at 24 h after ischemic injury (Fig. 3). Tan IIA (10 mg kg^{-1}) did not modify the water content significantly, although there was a significant reduction of infarct size.

Tan IIA reduced the Evans blue extravasation in the ischemic hemisphere

To clarify the possible protective mechanism for Tan IIA on ischemic brain injury, the effect of Tan IIA on BBB permeability was assessed with EB extravasation in the ischemic brain tissue. As shown in Fig. 4, the Evans blue dye content of the ischemic hemisphere in the vehicle group was significantly higher than that of the sham-operated group ($P < 0.05$). In the Tan IIA group, EB was significantly reduced compared with that of the vehicle groups in a dose-dependent manner ($P < 0.05$).

Tan IIA suppressed the expression of ICAM-1 proteins after ischemic injury

ICAM-1 leads to vascular inflammation and is considered to be closely related in the process of BBB injury induced by ischemia and reperfusion. As shown in Fig. 5, the expression of ICAM-1 significantly increased after ischemic injury. Tan IIA treatment resulted in a significant decrease in ICAM-1 expression in a dose-dependent manner ($P < 0.05$).

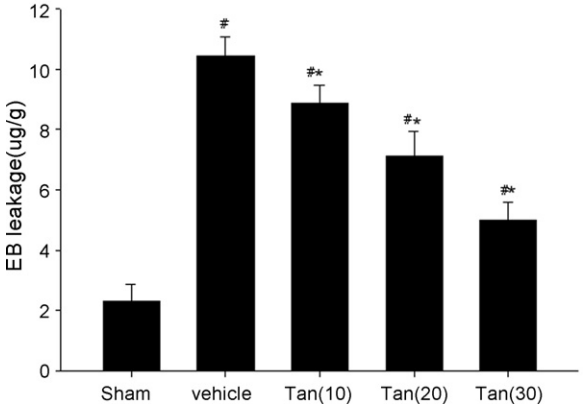

Fig. 4. Effects of Tan IIA on blood–brain barrier permeability after MCAO. The BBB permeability was assessed with EB extravasation. Rats were pretreated with indicated concentrations of Tan IIA (mg kg^{-1}) after MCAO. Values are shown as mean ± SD, $n = 6$, $^{*}P < 0.05$ *vs* vehicle group, $^{\#}P < 0.05$ *vs* sham group.

Tan IIA blocked the expression of MMP-9 after ischemic injury

MMP-9 is highly associated with BBB disruption. As shown in our data (Fig. 5), there was a low level of MMP-9 expression in the sham-operated group. After ischemic brain injury, the expression of MMP-9 increased significantly. Tan IIA treatment resulted in a significant decrease in MMP-9 expression.

Tan IIA inhibited the degradation of tight junction proteins after ischemic injury

We studied two major proteins involved in the tight junctions of BBB, ZO-1 and Occludin. Our data showed that both ZO-1 and Occludin were significantly degraded in the ischemic hemisphere. However, loss of ZO-1 and Occludin protein was successfully reversed after Tan IIA treatment (Fig. 5).

Discussion

In the present study, we demonstrated that Tan IIA treatment reduced brain infarct area, water content in the ischemic hemisphere. Tan IIA also significantly decreased BBB permeability to Evans blue, which was associated with a decrease in the expression of ICAM-1, MMP-9, inhibition of the degradation of tight junction proteins (Tjps) ZO-1 and Occludin. These results indicated that Tan IIA's most effective protection was on the BBB.

ICAM-1 is the best characterized cell surface adhesion molecule which is constitutively expressed on brain microvascular endothelial cells (BMVECs). Adhesion of leukocytes to endothelium mediated by ICAM-1 is considered to be closely related with the process of BBB injury induced by ischemia and reperfusion (Dietrich 2002). *In vitro* studies has shown that Tan IIA can markedly inhibited the production of NO, IL-1beta and TNF-α and suppressed the expression of iNOS in activated RAW 264.7 cells (Jang et al. 2003). As adhesion molecules are produced in response to these inflammatory stimuli. These responses of inflammatory stimuli to Tan IIA exposure would be expected to lead to inhibition of ICAM-1. Previous report has showed that Salvia miltiorrhiza root extract can reduce the expression of ICAM-1 and release of proinflammatory mediators in ischemia injury models effectively (Han et al. 2008). As a major active ingredient of SM extract, Tan IIA can protect vascular endothelial cells from ischemia injury (Lin et al. 2006). The protective effect of Tan IIA on endothelial cells is also supported by its inhibition on the ICAM-1 expression levels as demonstrated in this study.

BBB is a highly specialized BMVECs structure, which forms a unique, tightly interconnected, cellular monolayer, which are characterized by the presence of Tjps. The major molecular components of Tjps include transmembrane proteins, Occludin and cytoplasmic accessory proteins ZO-1, disruption of which will result in the leakage of BBB (Michel et al. 2002). Tjps are targets of MMPS in the influence on the BBB permeability after brain ischemia or hypoxia (Yang et al. 2007). MMPs play an important role in the degradation of the basal lamina and the invasion of endothelial cells. Proteolytic enzymes such as MMPs, in particular MMP-9, are highly sensitive to the microenvironment and respond to inflammatory cytokines *in vivo*, which result in BBB injury in cerebral ischemia (Svedin et al. 2007). To further understand the mechanism of action of the Tan IIA on the BBB. We also used Western blotting to analyze the levels of ZO-1, Occludin, MMP-9 expression. Our results showed that there was a significant MMPs activation led to BBB opening in the brain via Tjps degradation, resulting in reduction of ZO-1 and Occludin expression after ischemia injury. However, Tan IIA restored these proteins expression in a dose-dependent manner. Protection by Tan IIA against the ischemia/reperfusion-mediated

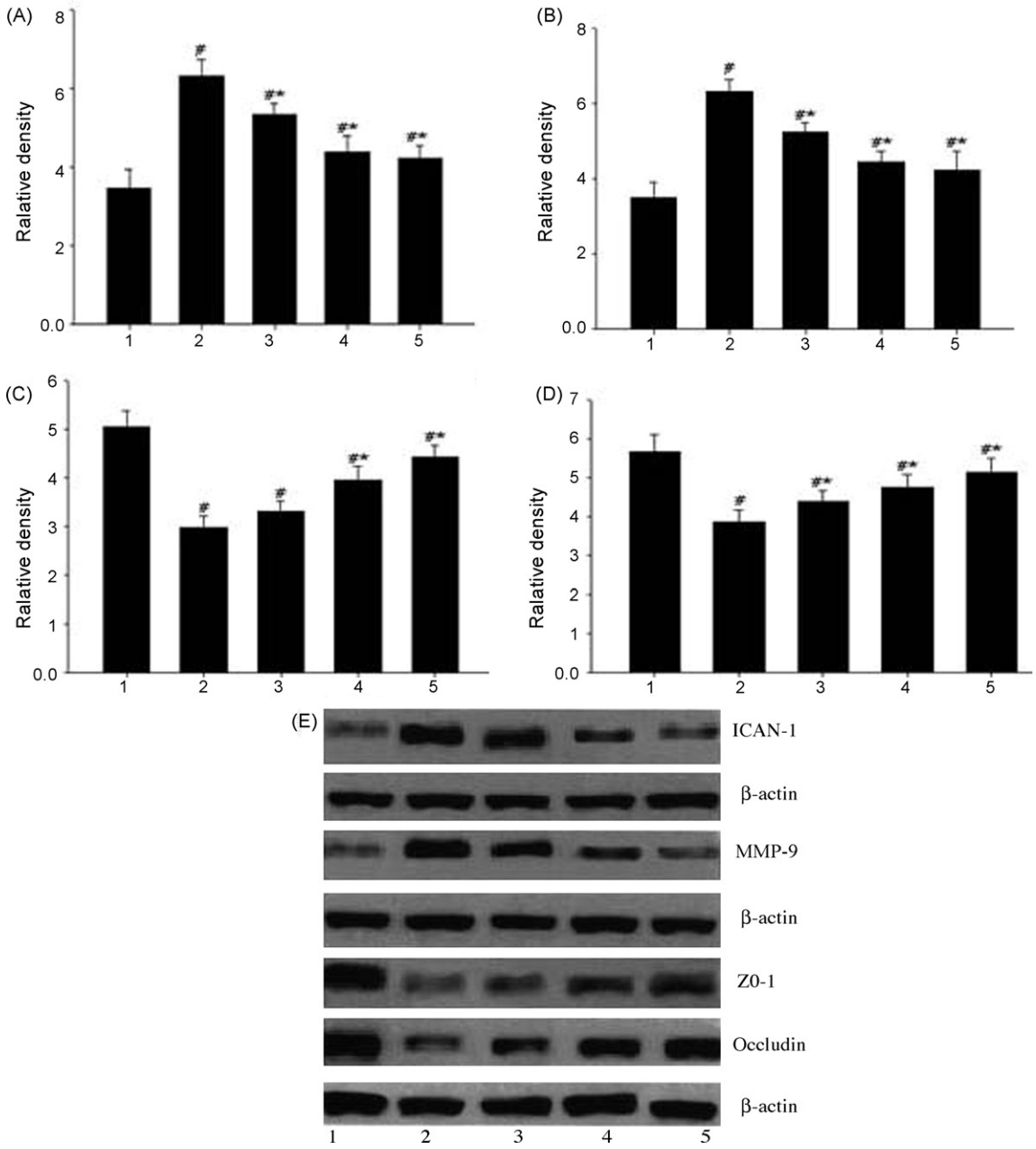

Fig. 5. Effects of Tan IIA on protein expression of ICAM-1 and MMP-9, ZO-1, Occludin in brain tissue. Protein levels were examined by Western blotting analyses in the presence or absence of pretreatment with indicated concentrations of Tan IIA after MCAO. Lane 1: sham; Lane 2: vehicle; Lane 3: Tan IIA (10 mg kg^{-1}); Lane 4: Tan IIA (20 mg kg^{-1}); Lane 5: Tan IIA (30 mg kg^{-1}). Values are shown as mean ± SD. $n = 6$, $^{*}P < 0.05$ *vs* vehicle group, $^{\#}P < 0.05$ *vs* sham group. (A) ICAM-1; (B) MMP-9; (C) ZO-1; (D) Occludin.

loss of ZO-1 and Occludin suggests that Tan IIA protects endothelial tight junctions and therefore keeps the BBB intact. Accordingly, an assessment by the EB extravasation method showed reduced BBB leakage in the Tan IIA treatment group. The results were further supported by decreased edema/water content in the ipsilateral side of the Tan IIA treatment group.

In conclusion, our results illustrated that Tan IIA was effective for attenuating the extent of brain edema formation in response to ischemia/reperfusion injury in rats, partly by Tan's protective effect on the BBB. Our results may have implications in the treatment of brain edema in cerebral ischemia.

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