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Tanshinone IIa Alleviates the Biochemical Changes Associated with Hypoxic Ischemic Brain Damage in a Rat Model

Mingyan Hei,* Yali Luo, Xiaochun Zhang and Furong Liu
Department of Pediatrics, The 3rd Xiangya Hospital of Central South University, Changsha, Hunan, China

This study aimed to investigate the effects of Tanshinone IIa (TanIIa) on the biochemical changes associated with hypoxic ischemic brain damage (HIBD) in a rat model. Neonatal SD rats were randomized into normal control, HIBD and TanIIa + HIBD groups. At different time points after HIBD, TanIIa was given at 1 μg/g. The intracellular free calcium concentration and the expression of phospho-NR1 S897 was determined. The intracellular free calcium concentration in the HIBD group was significantly increased. The induction of intracellular free calcium concentration in the TanIIa + HIBD group was less than that in the HIBD group. Large amounts of phospho-NR1 S897 positive cells were distributed in the cortex in the normal control group; the number of phospho-NR1 S897 positive cells in the ipsilateral cortex was dramatically decreased at 24 h after HIBD. Both the number of phospho-NR1 S897 positive cells and the FITC fluorescent density in the HIBD + TanIIa group were less than those in the normal control group at every time point after HIBD, but more than those in the HIBD group. TanIIa alleviated the down-regulation of phospho-NR1 S897 and the elevated intracellular free calcium concentration in the cerebral cortex in the HIBD model. TanIIa could exert a neuroprotective effect through affecting NMDA receptor expression, inhibiting calcium transportation and decreasing the intracellular free calcium concentration.

Keywords: Tanshinone IIa; hypoxia-ischemia; brain damage; phospho-NR1 S897; rat.

INTRODUCTION

Hypoxic ischemic brain damage (HIBD) is a common disease in infants. During HIBD, the brain cells undergo a series of biochemical and pathophysiological changes, such as activation of *N*-methyl-D-aspartate (NMDA) receptors and intracellular free calcium concentration. There is no specific medication for HIBD, but some herbs, such as the root of red-rooted *Salvia miltiorrhiza*, has been found to exert neuroprotective effects on patients and has been used as a complementary and alternative medicine in the clinic (Liu *et al.*, 2010a, 2010b, 2010c; Lam *et al.*, 2003; Liu *et al.*, 2010a, 2010b, 2010c). However, the underlying mechanism by which these herbs exert the effects remains unclear. Tanshinone IIa (TanIIa) is a compound isolated from *Salvia miltiorrhiza*, which is a phenanthrene-quinone derivative that has a potent effect (Fu *et al.*, 2007). Research has indicated that TanIIa has a protective effect on acute myocardial infarction in a rat model (Ren *et al.*, 2010). TanIIA modulates the cell response to hypoxia by inhibiting calcium influx and can decrease the intracellular free calcium concentration (Wang *et al.*, 2010), which is considered to be the major pathway of the cell-protective effect of TanIIA. The effect of TanIIA on the intracellular free calcium concentration may be through regulation of the NMDA receptor, since the NMDA receptor is a calcium channel (Sun *et al.*, 2010). The present study investigated the effect of TanIIa on phosphorylated NMDA receptor 1 expression and intracellular free calcium concentration ($[Ca^{2+}]_i$) in a hypoxic ischemic brain damage (HIBD) neonatal SD rat model. The mechanism by which TanIIa has a neuroprotective role in HIBD was also discussed.

MATERIALS AND METHODS

All animals received care in compliance with the UK Animals (Scientific Procedures) Act 1986 and associated guidelines, the EEC Directive of 1986 (86/609/EEC) or the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23; revised 1978), and the study was approved by the Ethics Committee of Central South University, Hunan, China.

Animal model and TanIIa injection. A total of 240 new born (7 day old, weight 13.6 ± 2.3 g) SD rats were randomly assigned to control, HIBD and HIBD + TanIIa groups. Half of the rats were used for measuring the intracellular free calcium concentration $[Ca^{2+}]_i$, and the other half of the rats were killed for immunohistochemistry (IHC) staining. The time points for experiments were 3 h, 6 h, 12 h and 24 h after HIBD.

The HIBD animal model was established according to the Rice-Vannucci method (Rice *et al.*, 1981). Briefly, rats were anesthetized with ether and the right common carotid artery was surgically freed and split. The rats were

* Correspondence to: Mingyan Hei, Department of Pediatrics, The Third Xiangya Hospital of Central South University, 138 Tongzi Road, Changsha, Hunan, China 410013.
E-mail: heiming_yan@yahoo.com.cn

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Table 1. The effect of TanIIa on intracellular free calcium concentration

Group	*n*	Time after HIBD (h)			
		3	6	12	24
Control	10	1.003 ± 0.201	0.996 ± 0.191	0.987 ± 0.199	0.936 ± 0.191
HIBD	10	1.336 ± 0.222[a]	1.245 ± 0.198[a]	1.233 ± 0.239[a]	1.412 ± 0.161[a]
TanIIa + HIBD	10	1.239 ± 0.194[a]	1.249 ± 0.211[a]	1.216 ± 0.199[a]	1.131 ± 0.051[b]

[a] Control vs HIBD, $p < 0.05$.
[b] TanIIa + HIBD vs HIBD, $p < 0.05$.

then put back to the mother for 1 h and exposed to hypoxia conditions (8% O_2/92% N_2 atmosphere) for 2.5 h at 34 °C. Immediately after hypoxia, which was set as the zero reference time point, the rats were injected with TanIIa at a dose of 1 μg/g weight. TanIIa injection solution was purchased from Shanghai First Biochemical Drug Co. Ltd (10 mg/2 mL, lot number 060302). Additional TanIIa was injected every 12 h to maintain the serum level.

Measurement intracellular free calcium concentration $[Ca^{2+}]_i$. Surgically dissected cerebral cortex was trypsinized, resuspended and centrifuged to make a cell suspension at 2×10^7 cells/mL. Then Fura-2 AM (Sigma) was added to the cell suspension to make a final concentration of 5 μM and incubated at 37 °C for 45 min (Rice *et al.*, 1981; Ahmed *et al.*, 2002). The Fura-2 AM fluorescence was measured using a Hitachi F-4500 scanner. F_{max} and F_{min} were obtained by adding 0.1% Triton X-100 and 0.25 mM $CaCl_2$ +10 mM EDTA, respectively. The fluorescence ratio (R) activated at 340 nm and 380 nm was calculated. Then the $[Ca^{2+}]_i$ was measured using the following formula: $[Ca^{2+}]_i$ (nM) = K_d B $[(R - R_{min})/(R_{max} - R)]$ (K_d is the dissociation constant 224, $B = R_{min}/R_{ma}$). The ratio of $[Ca^{2+}]_i$ between the lesion (right side) to the control (left side) was calculated for each rat (Levin *et al.*, 1991).

Immunohistochemistry (IHC) staining. At 3 h, 6 h, 12 h and 24 h after HIBD, the rats ($n = 10$ each time point and each group) were subjected to cardiac perfusion. The brain was dehydrated by using 30% sucrose solution and cut into 10 μm slices. The slices were blocked using 3% BSA + 0.1% Triton-X 100 + 15% NGS at room temperature, and incubated with primary antibody (rabbit phospho-NR1 S897 antibody, 1:500, Upstate Biotechnology, Lake Placid, NY) at 4 °C overnight. For negative controls, non-immune serum was used. Then the slices was washed with PBS and incubated with secondary antibody (sheep anti rabbit IgG FITC, 1:200, Pharmingen) for 1 h at room temperature in the dark. Next, after washing with PBS, 0.5 g/mL DAPI was used for staining and the results were observed under microscopy (Leica DMRB microscope). The green fluorescence intensity was measured by averaging three randomly selected fields under the microscope. At 24 h after HIBD, which was the time point that the most significant fluorescence was observed, the positively stained cells in the horizontal section of the hippocampus in HIBD and HIBD + TanIIa groups were counted using StereoInvestigator 3.0 software.

Statistical analysis. The data were presented as mean ± SD and analysed using SPSS14.0 software. One-way ANOVA was used for comparison between the different groups. Fisher's least significant difference (LSD) or Student–Newman–Keuls tests were used. A value of $p < 0.05$ was considered statistically significant.

RESULTS

The effects of TanIIa on $[Ca^{2+}]_i$

The $[Ca^{2+}]_i$ was similar between the right and left sides of the hemisphere, with a ratio of 1:1 (Table 1). In the HIBD group, the $[Ca^{2+}]_i$ was higher in the lesion side, with the most significant differences being observed at 3 h and 24 h after HIBD ($p < 0.05$). In the HIBD + TanIIa group, the $[Ca^{2+}]_i$ was higher than that of the control group, with significant differences being observed at 3 h, 6 h and 12 h after HIBD ($p < 0.05$); however, the difference was not significant at 24 h ($p > 0.05$). In addition, the elevation level of the $[Ca^{2+}]_i$ was lower in the TanIIa + HIBD group than that in the HIBD group. Particularly at 24 h, $[Ca^{2+}]_i$ in the HIBD + TanIIa group was significantly decreased by 24.9% compared with the HIBD group ($p < 0.05$).

Phospho-NR1 S897 expression in the cerebral cortex of normal neonatal rats

Phospho-NR1 S897 was expressed in the cerebral cortex, forming a ubiquitous pattern. The cells were of normal shape and organized. Phospho-NR1 S897 was mainly expressed in the cell plasma (Fig. 1).

Phospho-NR1 S897 expression in the cerebral cortex of HIBD rats and HIBD rats injected with TanIIa

In the HIBD group, the phospho-NR1 S897 positive cell number was significantly less than that of the normal group. Notably, phospho-NR1 S897 positive cell numbers were gradually decreased at 3 h, 6 h, 12 h and 24 h (Fig. 2) after HIBD, compared with the zero time point. In the HIBD + TanIIa group, the lesion side (ipsilateral) of the cerebral cortex had slightly fewer phospho-NR1 S897 positive cells, compared with the normal side (contralateral) of the cerebral cortex; but had more phospho-NR1 S897 positive cells, compared with the HIBD group (Fig. 2). The differences between the HIBD + TanIIa group and the HIBD group were significant at 3 h and 24 h after HIBD (Fig. 2). When compared between groups, the green fluorescence intensity of the HIBD + TanIIa group was lower than that of the control group, but significantly higher than that of the HIBD group. After 24 h of HIBD, the

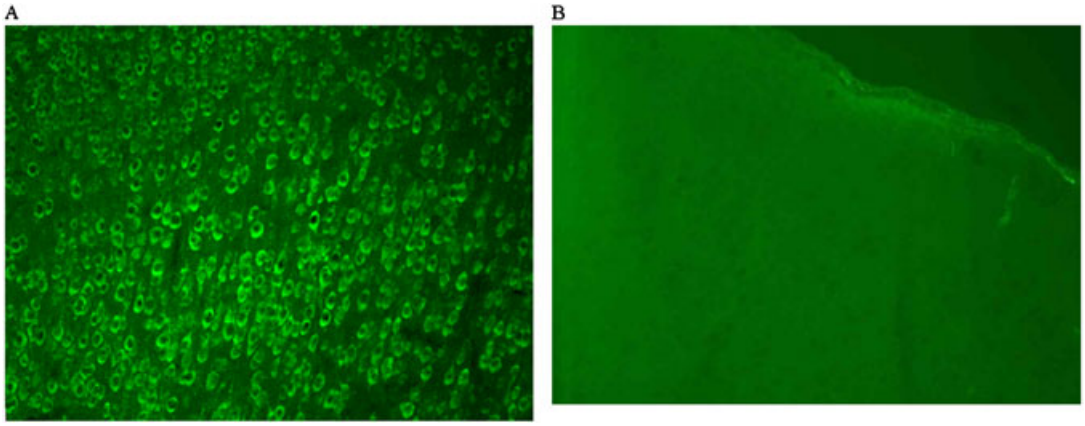

Figure 1. (A) Phospho-NR1 S897 expression in the cerebral cortex of normal neonatal rats (200×). (B) Non-immune serum as negative control.

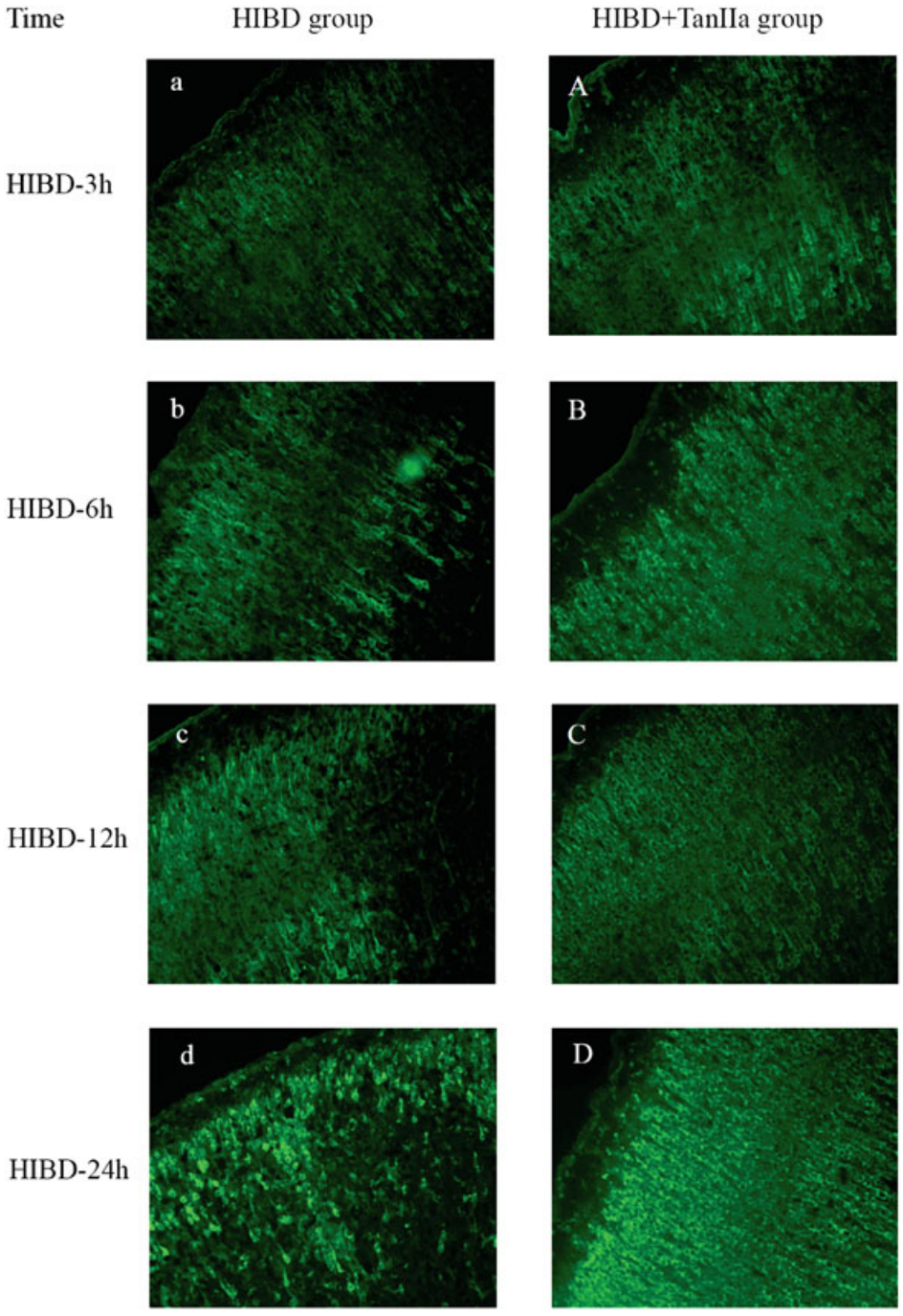

Figure 2. Phospho-NR1 S897 expression in the cerebral cortex of HIBD rats and HIBD rats injected with TanIIa (200×). (a, b, c, d), 3 h, 6 h, 12 h, 24 h after HIBD, respectively; (A, B, C, D), 3 h, 6 h, 12 h, 24 h after HIBD + HIBD, respectively.

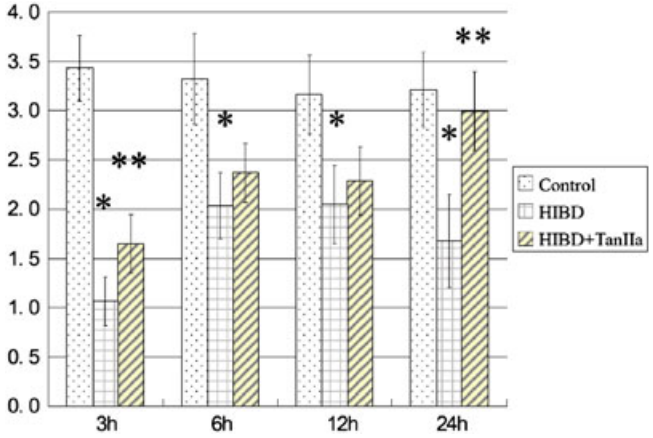

Figure 3. Green fluorescence intensity of phospho-NR1 S897 in the cerebral cortex at different time points after HIBD. A Leica DMRB microscope was used for image analysis. Three fields were selected and images obtained using a Leica DMRB microscope. The GFP intensity averaged for the results is presented. *$p < 0.05$; **$p < 0.01$.

Table 2. The number of phospho-NR1 S897 positive cells in the lesion side and normal side of cerebral cortex in HIBD and HIBD + TanIIa groups at 24 h after HIBD (mean ± SD)

Group	*n*	Phospho-NR1 S897 positive cells		
		HIBD lesion side	HIBD normal side	Ratio
Control	10	22347 ± 3689	21994 ± 5417	1.008 ± 0.044
HIBD	10	483 ± 94	19341 ± 3067	0.405 ± 0.058
TanIIa + HIBD	10	16033 ± 3629[a]	20153 ± 3794	0.783 ± 0.048[a]

[a] TanIIa + HIBD vs HIBD, $p < 0.05$.

difference between the HIBD and the HIBD + TanIIa groups was the most significant ($p < 0.05$) (Fig. 3). Moreover, the cell count results also showed that the number of phospho-NR1 S897 positive cells in the lesion side cerebral cortex of the HIBD+TanIIa group was significantly higher than that in the HIBD group ($p < 0.05$) (Table 2).

DISCUSSION

TanIIa ($C_{19}O_3H_{20}$, molecular weight 296) is a compound isolated from *Salvia miltiorrhiza* (Wang *et al.*, 2010), which has been used in Traditional Chinese herbs as the most critical component for quality control (Hazra *et al.*, 2004). Some animal studies have indicated that TanIIa has a neuroprotective effect in a hypoxic brain damage rat model (Xia *et al.*, 2005) and an ischemic brain damage mouse model (Dong *et al.*, 2009). It has also been shown that TanIIa is an effective medication for ischemic stroke (Liu *et al.*, 2010a, 2010b, 2010c). This study investigated the effects of TanIIa on phospho-NR1 S897 expression and intracellular free calcium concentration in the hypoxic ischemic brain damage neonatal SD rat model. The results showed that TanIIa alleviated the HIBD-resulting down-regulation of phospho-NR1 S897 and the HIBD-induced $[Ca^{2+}]_i$ elevation in the cerebral cortex. These results indicate that the neuroprotective effect of TanIIa was through affecting NMDA receptor expression and decreasing the intracellular free calcium concentration.

After HIBD, the brain cells undergo a series of pathophysiological changes, including the activation of aspartic acid receptor, calcium concentration, mitochondria damage and apoptosis (Johnston, 2005). In this study, $[Ca^{2+}]_i$ was increased at every time point after HIBD, which confirms the pathophysiological changes of HIBD. By contrast, the induction of $[Ca^{2+}]_i$ in the HIBD + TanIIa group was inhibited at every time point after HIBD, indicating that TanIIa can alleviate the intracellular free calcium concentration, a critical pathway of cell damage and apoptosis. Therefore, the neuroprotective effect of TanIIa was possibly through decreasing the intracellular free calcium concentration.

The NMDA receptor, which is composed of NR1 and NR2, is an important calcium channel. Activation of NR1 is correlated with phosphorylation and dephosphorylation of the NR1 S897 site. It was shown that phospho-NR1 S897 was highly expressed in the cerebral cortex of normal neonatal rats. This indicates that most NR1 S897 sites are phosphorylated in normal physiological conditions, which is required for maintaining the normal physiological function of the neurons. At 3 h to 24 h after HIBD, phospho-NR1 S897 expression was significantly down-regulated, indicating that dephosphorylation of the NR1 S897 site contributes to the brain damage. TanIIa, as a neuroprotective drug, can inhibit the HIBD-induced phospho-NR1 S897 dephosphorylation. Moreover, TanIIa can also decrease the HIBD-induced intracellular free calcium concentration. When comparing the effects of TanIIa at different time points, it was shown that phospho-NR1 S897 expression was more significantly increased in the HIBD+TanIIa than in the HIBD group at 3 h and 24 h, while the intracellular free calcium concentration in the HIBD + TanIIa group was inhibited only at 24 h compared with the HIBD group. These results suggest that the effect

of TanIIa on phospho-NR1 S897 preceded its effect on intracellular free calcium. Therefore, it is possible that the neuroprotective effect was through a pathway involving three steps: (1) inhibition of phospho-NR1 S897 dephosphorylation, (2) inhibition of internal calcium transportation, (3) decreasing $[Ca^{2+}]_i$.

Some other investigators also reported the effect of TanIIa in animal models. TanIIa was found to attenuate hypertrophy induced by angiotensin II in cultured neonatal rat cardiac cells, and to depress angiotensin II-induced cardiomyocyte hypertrophy through the MEK/ERK pathway (Takahashi *et al.*, 2002; Yang *et al.*, 2007). TanIIa also has a renoprotective effect in rats with chronic kidney disease (Ahn *et al.*, 2010). In addition, TanIIa can inhibit the effect of lipid peroxidation on ischemic reperfusion injury (Fu *et al.*, 2007). Ren *et al.* reported that TanIIa could attenuate inflammatory responses in rats with myocardial infarction by reducing MCP-1 expression (Ren *et al.*, 2010). Dong *et al.* also reported that TanIIa intraperitoneal injection could alleviate HIBD by increasing superoxide dismutase activity and by decreasing iNOS expression, indicating that the neuroprotective effect of TanIIa on HIBD is through inhibiting oxidative stress and free radical-mediated inflammation (Dong *et al.*, 2009).

In summary, it was demonstrated that TanIIa can alleviate HIBD-associated down-regulation of phospho-NR1 S897 and HIBD-induced $[Ca^{2+}]_i$ elevation in the cerebral cortex. The neuroprotective effect of TanIIa was through affecting NMDA receptor expression, inhibiting calcium transportation and decreasing the intracellular free calcium concentration.

Conflict of Interest

The authors have declared that there is no conflict of interest.

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