Research Report

Pentoxifylline attenuates TNF-α protein levels and brain edema following temporary focal cerebral ischemia in rats

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ABSTRACT

Cerebral edema is the most common cause of neurological deterioration and mortality during acute ischemic stroke. Despite the clinical importance of cerebral ischemia, the underlying mechanisms remain poorly understood. Recent studies suggest a role for TNF-α in the brain edema formation. To further investigate whether TNF-α would play a role in brain edema formation, we examined the effects of pentoxifylline (PTX, an inhibitor of TNF-α synthesis) on the brain edema and TNF-α levels in a model of transient focal cerebral ischemia. The right middle cerebral artery (MCA) of rats was occluded for 60 min using the intraluminal filament method. The animals received PTX (60 mg/kg) immediately, 1, 3, or 6 h post-ischemic induction. Twenty-four hours after induction of ischemic injury, permeability of the blood–brain barrier (BBB) and brain edema were determined by in situ brain perfusion of Evans Blue (EB) and wet-to-dry weight ratio, respectively. TNF-α protein levels in ischemic cortex were also measured at 1, 4, and 24 h post-ischemic induction. Twenty-four hours after induction of ischemic injury, permeability of the blood–brain barrier (BBB) and brain edema were determined by in situ brain perfusion of Evans Blue (EB) and wet-to-dry weight ratio, respectively. TNF-α protein levels in ischemic cortex were also measured at 1, 4, and 24 h after the beginning of an ischemic stroke by using an enzyme-linked immunosorbent assay method. The administration of PTX up to 6 h after occlusion of the MCA rats was occluded for 60 min using the intraluminal filament method. The animals received PTX (60 mg/kg) immediately, 1, 3, or 6 h post-ischemic induction. Twenty-four hours after induction of ischemic injury, permeability of the blood–brain barrier (BBB) and brain edema were determined by in situ brain perfusion of Evans Blue (EB) and wet-to-dry weight ratio, respectively. TNF-α protein levels in ischemic cortex were also measured at 1, 4, and 24 h after the beginning of an ischemic stroke by using an enzyme-linked immunosorbent assay method. The administration of PTX up to 6 h after occlusion of the MCA significantly reduced the brain edema. Moreover, PTX significantly reduced the concentration of TNF-α in ischemic brain cortex up to 4 h post-transient focal stroke (P<0.002). Finally, treatment by PTX led to a significant decrease in EB extravasations (P<0.001). Our data demonstrate that PTX administration up to 6 h after ischemia can reduce brain edema in a model of transient focal cerebral ischemia. The beneficial effects of PTX may be mediated, at least in part, through a decline in TNF-α production and BBB breakdown.

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1. Introduction

Brain edema is the most common cause of neurological deterioration and mortality during acute ischemic stroke (Zador et al., 2009). Ischemic brain edema is initially cytotoxic because of disturbances in cell membrane (Zador et al., 2009). Vasogenic cerebral edema is the most common type of brain edema, resulting from a disruption in blood–brain barrier (BBB) (Strbian et al., 2008; Zador et al., 2009). Cerebral edema usually begins to develop shortly after the onset of ischemia and
increases intracranial pressure (ICP) and potentially leads to brain ischemia, herniation, and finally death (Manley et al., 2004). Despite its clinical importance, pathophysiology of cerebral edema remains poorly understood. Currently, the clinical treatment of brain edema is based mainly on injection of hyperosmolar agents (e.g. mannitol, hypertonic saline) in the attempt to draw water out of brain tissue and decrease ICP (Manley et al., 2004). However, new therapeutic strategies are needed to more effectively treat brain edema following neurological disorders.

Recently, several lines of evidence support a role for tumor necrosis factor-α (TNF-α) in the pathophysiology of brain edema (Yang et al., 1999; Gregersen et al., 2000; Kimura et al., 2003; Hosomi et al., 2005). TNF-α has been demonstrated to participate in opening of the BBB and edema formation, which follows cerebral ischemic injury (Yang et al., 1999; Hosomi et al., 2005), brain trauma (Shohami et al., 1997), sepsis (Tsao et al., 2001), and bacterial meningitis (Ramilo et al., 1990).

Pentoxifylline (PTX), a phosphodiesterase inhibitor, rapidly penetrates into the BBB following systemic administration (Watkins et al., 2003; Fujimoto et al., 1976) and efficiently inhibits TNF-α mRNA synthesis (Nataf et al., 1993; Hong et al., 1995; Shohami et al., 1996; Hoie et al., 2004). Our previous studies showed that PTX has a neuroprotective effect in a model of focal cerebral ischemia (Nekoeian et al., 2005; Vakili and Zahedi Khorasani, 2007a). An earlier study has demonstrated no effect of PTX on brain edema 6 h after the middle cerebral artery occlusion (MCAO) in hypertensive rats (Johansson and Olsson, 1989). In addition, one study has reported that PTX significantly lowered cerebral edema, along with the reduced levels of TNF-α by 80%, in closed head injury in rats (Shohami et al., 1997).

To the best our knowledge, only a few studies have addressed the effects of the PTX on cerebral edema following experimental brain injuries and, to date, no study has investigated its effects on BBB integrity. Hence, we investigated the influence of PTX on brain edema, BBB permeability, and TNF-α protein levels in focal cerebral ischemic stroke that was induced by temporary occlusion of the middle cerebral artery in rats.

2. Results

2.1. Cerebral blood flow

Regional cerebral blood flow (rCBF) was reduced to less than 20% of baseline in both saline and PTX treated groups. PTX did not significantly change rCBF during MCAO or reperfusion as compared with the control group (Fig. 1).

2.2. Time-course effects of PTX on brain water content

Measurement of brain water content (BWC) was used to evaluate brain edema. BWC in the left and right hemispheres of the sham-operated rats was 78.8%±0.13% and 78.7%±0.14%, respectively. Induction of focal cerebral ischemia in the saline group significantly increased BWC of ischemic hemisphere (right) to 82.99%±0.35% (P<0.001). As shown in Fig. 2A, PTX administration at beginning (80.6%±0.24%), 1 h (80.8%±0.3%), 3 h (81.03%±0.25%), or 6 h (80.78%±0.28%) after MCAO significantly reduced post-ischemic increase of BWC as compared with the saline group (P<0.001). In addition, the repetitive injections of PTX immediately, 1 h, 3 h, 6 h after MCAO, significantly reduced the percentage of BWC (edema) (81.5%±0.32%) as compared with the control group (82.99%±0.35%) (P<0.001). Furthermore, no significant difference in BWC was found in non-ischemic hemisphere between the groups (Fig. 2B).

Fig. 1 – Regional cerebral blood flow (rCBF; % from baseline) before and during MCAO and after reperfusion in the saline or PTX (60 mg/kg) treated groups.
2.3. Effect of PTX on BBB permeability

Evans Blue (EB) extravasation was used as a marker of BBB breakdown following cerebral ischemia. Induction of cerebral ischemia significantly increased the EB dye content of the ischemic hemisphere (right) ($P < 0.001$) in the saline group ($1.2 \pm 0.16 \mu g/g$ tissue) as compared with the sham group ($0.16 \pm 0.02 \mu g/g$ tissue; $P < 0.001$). Administration of PTX at beginning of ischemia significantly reduced the EB extravasations ($0.67 \pm 0.04 \mu g/g$ tissue; $P < 0.001$) into ischemic brain as compared with the saline group ($1.2 \pm 0.16 \mu g/g$ tissue; Fig. 3). There are no significant difference between the EB content in left hemisphere (non-ischemic) in the sham ($0.18 \pm 0.02 \mu g/g$ tissue), saline ($0.20 \pm 0.01 \mu g/g$ tissue), and PTX treated ($0.16 \pm 0.04 \mu g/g$ tissue) groups.

2.4. Effect of PTX on TNF-α protein levels in ischemic cortex

Induction of focal cerebral ischemia increased TNF-α protein levels significantly at 1 h ($5.45 \pm 0.60 \text{ pg/mg protein}$), 4 h ($14.48 \pm 2.1 \text{ pg/mg protein}$), and 24 h ($6.63 \pm 0.81 \text{ pg/mg protein}$) following ischemia in the right ischemic cortex in the saline treated groups as compared with the sham-operated group ($19 \pm 0.28 \text{ pg/mg protein}$) ($P < 0.001$; Fig. 4). TNF-α protein levels in cortical ischemic tissue of the saline groups were significantly increased 1 h after MCAO, reached to peak at 4 h post-ischemia, and reduced to baseline 24 h after MCAO. Systemic administration of PTX at 1 h ($2.60 \pm 0.66 \text{ pg/mg protein}$) and 4 h
(3.27±0.71 pg/mg protein) but not 24 h (4.15±1.27 pg/mg protein) after MCAO significantly reduced the TNF-α amount in cortical ischemic tissue as compared with the saline group (P=0.002; Fig. 4). These results indicate that PTX reduced TNF-α production in cortical ischemic tissue up to 4 h after MCAO.

3. Discussion

Our results indicated that systemic injection of PTX (60 mg/kg) up to 6 h following focal stroke reduces brain edema. This interesting finding indicates that the anti-edematous effects of PTX persisted for at least 6 h after post-transient focal stroke. This result is in accordance, to some extent, with our recent work showing a neuroprotective effect of PTX on cortical brain ischemic damage, which lasted for at least 3 h post-transient focal stroke in rats (Vakili and Zahedi Khorasani, 2007a). Furthermore, our results are consistent with other study showing an anti-edematous effect of PTX (20 mg/kg) against brain edema following close head injury in rats (Shohami et al., 1997). However, our present finding is in disagreement with an earlier report demonstrating no beneficent effect of PTX on brain edema in a permanent model of focal ischemia (Johansson and Olsson, 1989). Although the reason for this discrepancy is not clear, it may be related to the experimental design and methodology. In their study, PTX continuously infused with a dose of 0.30 mg/kg per minute starting 30 min after MCAO and brain edema was measured by specific gravity 6 h later in spontaneously hypertensive rats. We measured, however, the effects of PTX (60 mg/kg) on brain edema 24 h after transient MCAO. We chose this time because previous studies have showed that brain edema reached its maximum 24 h after ischemia (Lin et al., 1993; Slivka et al., 1995) and brain trauma (Louin et al., 2006).

The duration of PTX action is limited by its short half-life (less than 1 h) (Fujimoto et al., 1976; Rocci et al., 1987). Thus, we tested whether multiple treatments of PTX can reduce brain edema more than a single injection. The animals were treated with PTX immediately, 1, 3, and 6 h after MCAO. The findings revealed no significant changes on the percentage of brain edema following multiple injections of PTX, suggesting that a single injection is sufficient enough to effectively reduce brain edema.

An enhancing effect of PTX on the cerebral blood flow has been reported in humans with cerebrovascular disorder (Bowton et al., 1989). Nevertheless, animal studies have failed to observe an effect of PTX on regional cerebral blood flow during ischemia or reperfusion in an experimental animal model (Johansson, 1986; Toung et al., 1994). In this study, we did not observe any effect of PTX administration at initial of ischemia on regional cerebral blood flow before during MCAO. Thus, we can exclude the possibility that the observed anti-edematous effect of PTX is due to an increased cerebral blood flow during MCAO.

To determine the mechanism of the anti-edematous action of PTX, TNF-α concentration was measured 1 h, 4 h, and 24 h after MCAO in the saline and PTX treated animals. We found a transient increase in TNF-α concentration 1 h after ischemia, reached to a peak during 4 h, and returned to baseline at 24 h post-ischemia. This finding is consistent with a report that demonstrated that TNF-α reached to a peak 4 h after close head injury (Shohami et al., 1997). Moreover, we found that PTX attenuates TNF-α activity in ischemic cortex, suggesting a role for TNF-α in the formation of brain edema in acute phase of transient focal stroke. In support of this result, other studies demonstrated that TNF-α is involved in brain edema following cerebral ischemia (Megyeri et al., 1992; Yang et al., 1999; Gregersen et al., 2000).

PTX is currently used for the treatment of some vascular disorders (Jacoby and Mohler, 2004). In recent years, PTX has evoked new interest because it has been found to attenuate the synthesis of TNF-α and other pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-6 in vivo and in vitro (Han et al., 1990; Kambayashi et al., 1995; Lauterbach et al., 1995; Marcinkiewicz et al., 2000). Our results showed an attenuation of brain edema by PTX along with decrease of BBB permeability and TNF-α level in ischemic cortex. Several evidence demonstrated that TNF-α is activated during brain ischemia (Gregersen et al., 2000; Maddahi and Edvinsson, 2010). It causes damages to cerebral endothelial cells (Kimura et al., 2003) and increases BBB permeability (Megyeri et al., 1992; Yang et al., 1999) and thus contributes to brain edema formation. Therefore, we propose that the anti-edematous effects of PTX are more likely due to an inhibition of TNF-α synthesis and, consequently, the protection of the BBB against disruption.

Recent studies reported that pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-6 and activation of NF-κB play important roles in the pathogenesis of brain edema (Bemeur et al., 2010; Rama Rao et al., 2010). The fact that PTX is able to decrease the levels of other inflammatory cytokines such as IL-1 and IL-6 as well as the activation of NF-κB (Han et al., 1990; Kambayashi et al., 1995; Lauterbach et al., 1995; Marcinkiewicz et al., 2000) suggests that the beneficial effect of PTX, at least in part, may result from the inhibition of production of these cytokines and, consequently, their signaling pathways. In addition, several evidences indicated that free radicals and oxidants have important roles in the development of edema and BBB disruption in experimental cerebral ischemia (Kondo et al., 1997; Heo et al., 2005). The fact that PTX exhibits antioxidant and free radical scavenger activities (Bhat and Madyastha, 2001) suggests that these properties may underlie the anti-edematous effect of PTX. Further experiments are needed to clarify these possibilities.

In conclusion, our study showed that PTX treatment could reduce up to 6 h after the onset of focal cerebral ischemia brain edema. This effect may be mediated via the inhibition of TNF-α synthesis and, consequently, the protection of the BBB disruption. Potential therapeutic applications of PTX in stroke patients need further studies.

4. Experimental procedures

4.1. Animals

Adult male Wistar rats (320±10 g) were obtained from breeding colony of Semnan University of Medical Sciences (SUMS), Semnan, Iran. All rats were housed in cages in a 12-h light/dark cycle at 22–24 °C, with food and water ad libitum. All procedures were conducted in agreement with the National
Institutes of Health Guide for Care and Use of Laboratory Animals.

4.2 Middle cerebral artery occlusion and laser Doppler flowmetry

MCAO was induced by intraluminal filament method as described previously (Vakili et al., 2005). Briefly, animals were anesthetized with chloral hydrate (400 mg/kg ip) and the right common carotid (CCA) and external carotid arteries were exposed through a midline neck incision. A nylon thread (3-0) inserted into the internal carotid artery and gently advanced until laser Doppler flowmetry (LDF; Moor Instruments DRT4, England) showed a sharp decrease in the ipsilateral cerebral blood flow to less than 20% of baseline, indicating adequate occlusion of the MCA. After 60 min of MCAO, reperfusion was done by withdrawing the intraluminal filament for 23 h.

To monitor the regional CBF during cerebral ischemia, a laser Doppler flowmetry probe (Moor Instruments DRT4, England) was positioned in direct contact with the right temporal bone over the right common carotid (CCA) and external carotid arteries after the reperfusion. Animals were randomly divided into seven groups. Group 1 was sham-operated (n=8); Group 2 (n=8) was the control group which received saline (1 mL/kg) as control; and four groups that received PTX at the beginning (n=8), 1 h (n=8), 3 h (n=8), and 6 h (n=8) after MCAO, respectively. Because half-life of PTX is less than 1 h (Fujimoto et al., 1976; Rocci et al., 1987), one additional group was injected (PTX) repetitively at the beginning, 1 h, 3 h, and 6 h after MCAO. The investigator who performed animal surgery was blinded to the treatment of groups. Twenty-four hours after MCAO, all rats were killed and the brains were removed. Then, the pons and olfactory bulb were removed and the brains were weighted to obtain their wet weight (WW). Subsequently, brains were dried at 110 °C for 24 h to determine their dry weight (DW). BWC was calculated by using the following formula: (WW– DW)/WW×100.

4.3 Brain water content

PTX was administrated at a dose of 60 mg/kg as the most effective dose. This dose was chosen based on our pilot study using different doses of the PTX (15, 30, 60, and 120 mg/kg) in focal cerebral ischemia model.

Cerebral edema was evaluated by determining brain water content (BWC) (Vakili et al., 2007b). For therapeutic window study, animals were randomly assigned six different groups. Group 1 was sham-operated (n=8); Group 2 (n=8) was the control group which received saline (1 mL/kg) as control; and four groups that received PTX at the beginning (n=8), 1 h (n=8), 3 h (n=8), and 6 h (n=8) after MCAO, respectively. Because half-life of PTX is less than 1 h (Fujimoto et al., 1976; Rocci et al., 1987), one additional group was injected (PTX) repetitively at the beginning, 1 h, 3 h, and 6 h after MCAO. The investigator who performed animal surgery was blinded to the treatment of groups. Twenty-four hours after MCAO, all rats were killed and the brains were removed. Then, the pons and olfactory bulb were removed and the brains were weighted to obtain their wet weight (WW). Subsequently, brains were dried at 110 °C for 24 h to determine their dry weight (DW). BWC was calculated by using the following formula: (WW– DW)/WW×100.

4.4 Evaluation of blood-brain barrier permeability by using Evans Blue

BBB permeability was measured at 24 h after MCAO in 3 different groups: Group 1 was sham-operated (n=6), Groups 2 and 3 (n=8 in each group) received saline or PTX at beginning of MCAO, respectively. EB (2% in saline, 1 mL/kg) was injected from tail vein at beginning of reperfusion. In all of these groups, BBB permeability was evaluated by measuring EB extravasations (Kucuk et al., 2002; Vakili et al., 2007b). Briefly, 24 h after ischemia, rats were deeply anesthetized, chest wall was opened, and transcardially perfused with 250 mL saline solution to clear cerebral circulation of EB. After decapitation, the brains were removed and hemispheres were separated and weighed. Each hemisphere was placed in the 2.5 mL phosphate buffer saline, was homogenized, and then 2.5 mL of 60% trichloroacetic acid (Merck, Germany) was added to precipitate protein. Samples were centrifuged for 30 min at 3500 rpm. The supernatants were measured at 610 nm for absorbance of EB using a spectrophotometer (UV–visible– single beam, PD-303UV; Apel, Japan). The results were expressed as microgram per gram tissue of wet weight of brain tissue calculated against a standard curve.

4.5 Determination of TNF-α in cerebral cortex

Animals were randomly divided into seven groups. Group 1 was sham-operated (n=6) and Groups 2–4 were controls that received saline (1 mL/kg) at the beginning of ischemia and the levels of TNF-α in their ischemic cortex tissue were measured at 1 h (n=6), 4 h (n=6), and 24 h (n=6) after MCAO, respectively. Groups 5–7 (n=6 in each group) received PTX (60 mg/kg ip) at the beginning of ischemia and the levels of TNF-α in their ischemic cortex tissue were measured at 1 h, 4 h, and 24 h after MCAO, respectively.

The levels of TNF-α in the ischemic cortex tissue was detected using an enzyme-linked immunosorbent assay (ELISA) test as described previously (Hang et al., 2004). Briefly, animals were killed 1 h, 4 h, and 24 h after MCAO. The brains were removed and cerebral cortex, dissected. The cortex was immediately frozen and kept at −70 °C. The frozen brain tissue was homogenized using a homogenizer in 500 μl buffer containing phosphate buffered saline (PBS) pH 7.2 (1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mg/L pepstatin A, 1 mg/L aprotinin, 1 mg/L leupeptin) and centrifuged at 12,000g for 20 min at 4 °C. Afterwards, supernatant was collected and total protein was determined by Micro BCA Protein Assay Kit. The level of TNF-α in ischemic cortex tissue supernatant was measured using an ELISA kit (Diaclone, France) specific for rat TNF-α. The measurement of TNF-α was performed step-by-step based on the protocol booklet of ELISA kit. The TNF-α contents were expressed as pg TNF-α/mg total protein (Hang et al., 2004).

4.6 Statistical analysis

Data are presented as mean±SEM. Data were analyzed by non-parametric Kruskal–Wallis ANOVA on ranks followed by a Dunn’s test. Differences were considered significant at P<0.05 (SigmaStat 2.0; Jandel Scientific, Erkrath, Germany).

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