Reduction in ischemic brain injury following the administration of pentoxifylline after transient global ischemia/ reperfusion in a rat model

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Received: 8 April 2014  Accepted: 24 June 2014  Published: 14 March 2015

Abstract

Background: It is well known that the hippocampus, the CA1 Pyramidal cells in particular, is selectively vulnerable during global cerebral ischemia. Recently, it is observed that pentoxifylline has a neuroprotective effect. This study explored the pharmacological relationship between ischemia-induced cell death of the hippocampus and the efficacy of a vasodilator agent (pentoxifylline) in the prevention of delayed neuronal death.

Methods: This experimental study was performed on 4 groups: control, ischemia, experimental (200mg/kg pentoxifylline injection one hour prior to and one hour following ischemia) and vehicle (normal saline). Transient global ischemia was induced by bilateral common carotid arteries occlusion. To investigate the apoptotic bodies and caspase-3 activities as a central role in the execution phase of apoptosis, the brains were prepared for the TUNEL technique.

Results: Pentoxifylline administration limited apoptosis and caspase-3 activities in rats’ hippocampus. Our data showed no significant difference between the number of apoptotic bodies in the CA1 region of the hippocampus in the control and pentoxifylline-treated groups ($p = 0.994$). The results of one-way ANOVA revealed that ischemia significantly increased caspase-3 levels in the hippocampus ($p < 0.05$); however, the level of caspase-3 in pentoxifylline-treated rats was less than the ischemic group.

Conclusion: These results suggest that the neuroprotective effect of pentoxifylline (200mg/kg) may be accompanied by a reduction in ischemic damage within the CA1 region of the hippocampus in rats subjected to transient global cerebral ischemia.

Keywords: Cerebral ischemia, Reperfusion, Pentoxifylline, Hippocampus.


Introduction

Stroke due to ischemia is the third cause of death and the leading cause of permanent disability in adults’ worldwide. The leading pathogenic mechanisms of cerebral ischemia include energy failure, elevation of intracellular Ca\textsuperscript{2+} level, glutamate mediated excitotoxicity, release of reactive oxygen species (ROS), blood brain barrier disruption, spreading depression, inflammatory response and apoptosis (1-3). Due to the advances in intravascular techniques and thrombolytic agents, transient global cerebral ischemia is one of the most common...
Protective effects of pentoxifylline on ischemia/reperfusion-induced neurotoxicity

In this experimental study, the animals (n= 40) were randomly divided into four groups. The control group rats were anesthetized with pentobarbital sodium (40mg/kg) and received no additional treatments. Bilateral common carotid occlusion (20 minutes) followed by reperfusion were performed for the 3 remaining groups (ischemia, experimental and vehicle groups). The experimental group rats were treated as same as the ischemia group except for receiving intraperitoneal (IP) injections of 200 mg/kg PTX one hour prior to and one hour after ischemia. Rats in the vehicle group were treated as same as the ischemia group but they were given IP injections of normal saline (0.5ml) one hour prior to and one hour after ischemia. The animals were sacrificed after four days. All hippocampi were removed and prepared for the TUNEL method and Western blot analysis.

Methods

Sample

Adult male Wistar rats, 12-13 weeks-old that weighed between 250-300 g were acquired from the Pharmacology Department of Tehran University of Medical Sciences and were used for all experiments. The rats were housed in standard animal houses and kept on a 12-hour light/dark cycle. All animals were maintained in the animal house for at least five days prior to conduction of the experiments and allowed free access to food and water.

All study-related procedures were approved by the Ethics Committee for the Use of Experimental Animals of Tehran University of Medical Sciences.

PTX powder was gifted by Amin Pharmaceutical Co. (Esfehan, Iran).

Experimental Groups and Drugs:

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**Surgical Procedure**

We carefully performed transient, global ischemia. For bilateral occlusion, a ventral midline incision was made to expose both carotid arteries which were then gently isolated from the carotid sheath and vagus nerve. Each carotid artery was ligated for 20 minutes using Yashargil Aneurism microclips at a point just below the carotid bifurcation. During ischemia, the animals were monitored for body temperature, loss of righting reflex and unresponsiveness to gentle touch. Subsequently, the carotid arteries were released and inspected for immediate reperfusion by removal of the clips and restoration of blood flow. Animals were returned to their home cage following surgery and maintained separately for four days (96 hours) after which they were killed by decapitation (8).

**Histopathology**

The rats were deeply anesthetized with pentobarbital (40mg/kg, IP) and their brains were fixed by cardiac perfusion with 4% paraformaldehyde buffered with 0.1 mol/L phosphate buffer (pH 7.2) that contained 4% sucrose. For light microscopy, the brain tissues were quickly removed and further immersed in the same fixative for at least three days. The samples processed for the TUNEL technique were embedded in paraffin, cut into 3 µm thick coronal sections by a rotary microtome, and then placed on silane-coated glass slides.

**TUNEL Technique**

To detect apoptotic cells, TUNEL staining was performed using an In Situ Cell Death Detection Kit (Cat No: 11684817910, Roche, Mannheim, Germany) according to the manufacturer’s protocol. The sections were deparaffinized in xylol, rehydrated by a successive series of alcohol, washed in phosphate-buffered saline (PBS) and deproteinized (or permeabilized) by proteinase K (20μg/ml) (Cat No: P2308, Sigma-Aldrich) for 30 minutes at a room temperature. Then, sections were rinsed and incubated with 3% H2O2 in methanol for 10 minutes in the dark to block endogenous peroxidase (POD). Next, sections were allowed to incubate in the TUNEL reaction mixture for 60 minutes at 37°C in a humidified atmosphere, after which they were rinsed with PBS. The sections were visualized by a converter-POD for 30 minutes at 37°C in a humidified atmosphere in the dark, then rinsed with PBS, followed by the addition of 50-100 µl dianisobenzidine (DAB, Cat No: D8001, Sigma-Aldrich) substrate and an additional PBS rinse (17).

**Western Blot Analysis**

Fresh hippocampi were homogenized in ice-cold lysis buffer that contained tris HCl (50mM, pH 8.0), NaCl (150mM), Nonidet P-40 (1%), glycerol (10%), phenylmethylsulfonyl fluoride (10μl/ml), sodium deoxycholate (0.5%) and aprotinin (30μl/ml), in addition to a protease inhibitor cocktail (Roche Applied Science, Cat No: 11697498001). The homogenized hippocampi were subjected to centrifugation at 12000 g for 20 minutes at 4oC and the supernatant was collected. A total of 100 µg from the total protein of the supernatant was loaded onto each lane and electrophoresed on SDS-PAGE gels (10%). Proteins were transferred onto nitrocellulose membranes for one hour at a room temperature and were blocked with PBS that contained non-fat dried milk powder (5%) for two hours. The membranes were washed by tris buffer that contained Tween 20 (1%), and then were probed with a monoclonal anti-cas3 antibody (1:1000; Abcam, St. Louis, MO, USA, Cat No: AB32351) overnight after which a secondary anti-rabbit akp-linked antibody (1:10000; Abcam, St. Louis, MO, USA, Cat No: AB6796) was added for one hour at a room temperature; then the membranes were stained with BCIP/NBT. β-actin served as a positive control for protein loading, and a high range molecular weight standard was used to determine protein sizes. Results were evaluated by the UVIdoc.
Statistical Analysis
The results are given as means±SD. The Significant difference level was determined by one-way ANOVA, followed by Tukey’s Multiple Comparison test. Statistical significance was set at p≤ 0.05.

Results
TUNEL Staining for Apoptotic Neurons in the Hippocampus
The number of TUNEL-positive cells was significantly elevated in the CA1 region of the hippocampus in the ischemia group compared to the control group (p= 0.045). There was a significant difference between the control and vehicle groups (p= 0.02); however, no significant difference was observed between the control and PTX-treated groups (p= 0.994), meaning that apoptotic cells significantly decreased following PTX treatment. Figs. 1 and 2 demonstrate representative TUNEL-positive apoptotic cells and the relationship of PTX treatment on the numbers of apoptotic cell bodies in the CA1 region of the hippocampus.

Western Blot Technique
The level of caspase-3 enzymatic activity as an initiator factor of apoptosis was measured by Western blot. The levels of caspase-3 activity were markedly increased in the hippocampi of the ischemia group compared to the control group. Caspase-3 levels were elevated in the hippocampi from the vehicle group, whereas the PTX-treated group had lower levels compared to those of the ischemia group (Fig. 3). These results showed that ischemia significantly increased caspase-3 levels in the hippocampus (p< 0.05); however, the level of caspase-3 in the PTX-treated group was less than observed in the ischemia group.

Discussion
Brain damage from ischemia is divided into two phases, the first of which is acute damage that leads to neuronal cell death. The main pathophysiologic mechanisms of ischemia include energy metabolism disturbance, calcium overload, and the production of oxygen-free radicals, nitric oxide, neurotoxic excitatory amino acids and destruction of the blood brain barrier (BBB). The second phase is delayed neuronal damage (DND) in which cerebral is-
chemia-reperfusion stimulates an inflammatory reaction and apoptosis, and it is the main pathophysiologic mechanisms of DND (19).

Global ischemia-reperfusion injury is characterized by the deterioration of brain
tissue following reperfusion (20). Many patients suffer from various degrees of learning and memory dysfunction which is indicative of impairment in the hippocampus, the primary area of the brain that controls the formation of learned behaviors and memories (21). It has been reported that the hippocampus, the CA1 region in particular, is selectively vulnerable during global cerebral ischemia (22).

Many studies have demonstrated that apoptosis during ischemia/reperfusion (I/R) plays a major role in stroke-associated brain injury (23). Apoptosis or programmed cell death when compared with necrosis is associated with less inflammation (24). Apoptotic cells are phagocytized by macrophages before their membrane breaks down; thus, their intracellular enzymes are not released. Apoptosis is triggered by two pathways: the intrinsic pathway which involves the mitochondria and is activated by reactive oxygen species and the extrinsic pathway that is activated when ligands bind to their receptors. An example of the extrinsic pathway is tumor necrosis factor-alpha (TNF-α) that binds to TNF-receptors and the Fas-ligand that binds to Fas (25).

We aimed to evaluate the effects of postischemic treatment of PTX on ischemic damage and neurological deficits in the rat model of transient global cerebral ischemia. PTX at a dose of 200 mg/kg was selected based on our previous study. We have previously reported that pre-ischemic treatment of PTX at a dose of 200 mg/kg (IP) could attenuate spatial memory impairment (8).

The results of the current study are also consistent with those of another report which have demonstrated that postischemic treatment of PTX at a dose of 40 mg/kg (IP) caused a reduction in cortical damage in rats (26). Additionally, our findings were in agreement with preclinical studies that demonstrated treatment with PTX reduced neuronal damage following ischemia (27).

TNF-α is a pro-inflammatory cytokine that plays an important role in the events following ischemia. The level of TNF-α increases during certain pathological conditions such as infectious, as well as neurological, neurodegenerative and neurotoxic conditions (28).

Numerous researches have shown the expression of TNF-α in ischemic neurons after cerebral ischemia (29). TNF-α mRNA becomes upregulated within 30 minutes in the ischemic brain, reaching a peak at 6–12 hour post-ischemia (30, 31).

PTX is a phosphodiesterase inhibitor that penetrates the blood–brain barrier which inhibits the production of TNF-α and other pro-inflammatory cytokines (32). It is well-known that TNF-α and glutamate can act synergistically to induce neuronal cell death (31). TNF-α can block glutamate transporter activity and induce glutamate neurotoxicity, which appears to be an important mechanism for neurodegeneration associated with inflammation. Recently, a wide range of anti-inflammatory properties have been ascribed to PTX such as inhibition of neutrophil activation, attenuation of inflammatory mediator production and prevention of endothelial-leukocyte adhesion (33). Vakilli et al. have observed that PTX significantly reduced the concentration of TNF-α in an ischemic brain cortex up to 4 hours post-transient focal stroke (34). In this study, we have hypothesized that at least a part of the neuroprotective effects of PTX is possibly related to the inhibition of TNF-α synthesis. In addition, the neuroprotective effect of PTX might be related to its ability to increase cerebral blood flow and neutrophil inhibition (35), reduce the release of inflammatory mediators such as platelet-activating factors and prevent endothelial–leukocyte adhesion (36). Perhaps PTX has the ability to inhibit free radical generation.

The present study showed that PTX can prevent neuronal apoptosis and thus causing a decrease in the numbers of damaged neurons in the neural tissue. However, previous studies are about focal brain ischemia and gene expression in apoptosis. However, in global brain ischemia model, injury oc-
curs within selectively vulnerable areas such as the CA1 pyramidal neurons (as our study) and 2 vessel occlusion (common carotid artery occlusion) is a suitable model to evaluate histopathology consequences from brain ischemia (37). TNF-α activates the caspase protease family cascade reaction. The caspase family promotes apoptosis in mammalian cells. Caspase-3 is activated in the apoptotic cell both by extrinsic (dead ligand) and intrinsic (mitochondrial) pathways (38). A variety of extracellular signals activate caspase-8 through the Fas receptor pathway and caspase-9 via mitochondrial cytochrome C in cerebral ischemia reperfusion injuries. The activation of caspase-8 and caspase-9 then promote caspase-3, which in turn hydrolyzes cell-specific proteins and poly-ADP ribose polymerase (PARP), inducing apoptosis (39). Thus, caspase-3 activity and TUNEL staining are different measures of apoptosis.

Conclusion
Our studies showed that the global ischemia insult for 20 minutes caused abundant neuronal cell death and apoptosis in the hippocampal CA1 region. However, when the animals were treated with PTX, there were lower numbers of TUNEL-positive cells in the hippocampus CA1 region compared to the ischemia group.

We further noted that PTX treatment down-regulated the expression of cleaved caspase-3 in the hippocampus. Possibly, PTX has an anti-apoptotic effect in ischemia reperfusion injury. To clarify the neuroprotective mechanism of PTX, we investigated the expressions of key apoptosis-related molecules and determined that cleaved caspase-3 increased in the ischemia group following global cerebral ischemia. However, it significantly decreased in the PTX (200mg/kg) treatment group relative to the vehicle and ischemia groups.

Our results revealed that PTX improves the outcome after global cerebral ischemia. This effect appears to be related to the anti-apoptotic action of PTX.

Acknowledgments
This work was supported by a grant from the Cellular and Molecular Research Center of Iran University of Medical Sciences, Tehran, Iran. We express our appreciation to the Amin Pharmaceutical Company (Esfahan, Iran) for their gift of pentoxifylline.

Conflict of Interest
The authors declare that they have no conflict of interest.

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