Activation of inflammatory mediators and apoptosis biomarkers following endothelin-1 induced acute ischemia in rats

Fatemeh Farokhi-Sisakht a,b *, Saeed Sadigh-Eteghad a, Pouran Karimi a

aNeurosciences Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.
bDepartment of Neuroscience, Faculty of Advanced Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran.

Abstract

Objective: Specific animal models have been created to mimic ischemic stroke. Endothelin-1 (ET-1) is a vasoconstrictor peptide that is increasingly utilized as a preclinical method for inducing cerebral ischemia in rodents. In the present study, we investigated the changes of inflammatory and apoptosis biomarkers in the acute phase of ET-1-induced ischemia.

Materials and Methods: 15 male rats were randomly divided into 3 groups (n=5): Control, sham, and ischemia. Rats of sham and ischemic groups underwent stereotaxic intrahippocampal injection of saline and ET-1, respectively. The protein levels of Nuclear factor-kappa B (NF-κB), tumor necrosis factor-alpha (TNF-α), tumor necrosis factor-alpha receptor 1 (TNFR1), cytochrome c, B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), and cleaved caspase-3 was evaluated by western blotting at 24 hours after surgery.

Results: Our finding were the first to show that ET-1 markedly upregulated the inflammatory and apoptotic proteins except for NF-κB in the acute stage of ischemia.

Conclusion: These data demonstrate that ischemia with ET-1 triggers inflammatory and apoptosis responses, which may be involved in exacerbating the ischemic injury.

Keywords: Endothelin-1, Acute ischemic stroke, Inflammation, Apoptosis.
cytoplasm and participates in the formation of a macromolecular complex called the apoptosome. This complex activates caspase cascades leading to neuronal apoptosis [12].

Although there are some rodent focal ischemia models, the ET-1 model has been introduced as a simple and invasive technique for the induction of focal ischemic stroke in rodents [13]. ET-1 is a long-acting peptide that functions as a vasoconstrictor agent in the vascular bed [14]. Hence, the purpose of this study was to explore the effect of ET-1 on the inflammatory and apoptotic processes in the acute phase of ischemia in the brain tissue of rats.

Materials and Methods

Animals and study design
Fifteen adult male Wistar rats (8-9 weeks old) were obtained from the animal house of Tabriz University of Medical Sciences (TUOMS). Animals were maintained in a room under controlled conditions of light (a 12 h light/dark cycle) and temperature (25±2) with free access to food and water. The experimental protocols were performed in accordance with the institutional guidelines and confirmed by the TUOMS Ethics Committee (TBZMED.REC. 1396.627). A person who was blind to nature of study divided the animals into three groups with five rats in each; the control group, rats received no injection. Ischemia model was induced by intrahippocampal injection of the vasoconstrictor ET-1. In the sham group, injection of saline was performed through same method.

Establishment of the acute cerebral ischemic model
Animals were initially anesthetized with 4% isoflurane using an induction chamber and maintained at 1.5% isoflurane in 30% O2 and 70% NO, then placed in a stereotaxic apparatus. After dural opening, bilateral intrahippocampal injection of ET-1 (15 pmol, 0.5 μL; 0.1 μL/min, Sigma–Aldrich, St. Louis, MO, USA) was performed using a Hamilton syringe according to the following coordinates: anteroposterior (AP) = -3.8 mm, mediolateral (ML) = ±2.2 mm and dorsoventral (DV) = -2.7 mm based on the rat brain in stereotoxic coordinates [15-17]. The animals of sham group underwent same surgical method, except that the saline was injected.

Sampling
24 hours after the surgery, the animals were deeply anesthetized with ketamine plus xylazine (90 and 10 mg/kg, i.p.). The brains were carefully removed and, the whole hippocampus were separated, the tissues immediately frozen, and stored at −80 °C before molecular analysis.

Western blotting
The hippocampal tissue proteins in each group were extracted by Radio Immuno Precipitation Assay (RIPA) [150 mM NaCl, 1% sodium dodecyl sulfate (SDS), 0.01% ethylene glycol tetra acetic acid (EGTA), 0.05 mM Tris pH 8, and 0.1% protease inhibitor cocktail (Roche, USA)] buffer and measured by Bradford’s assay. Protein samples were fractionated on 12.5% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to a polyvinylidenedifluoride (PVDF) membrane (Bio-Rad, USA) and the membranes were blocked for 120 min with phosphate buffer saline plus 0.05% Tween 20 (PBS-T) solution containing 1% Bovine serum albumin (BSA) and polybutylene succinate-co-butylen terephthalate (PBST). Then, the membranes were incubated with primary antibody against NFκβ, TNFα, TNFR1, Bax, Bcl-2, caspase-3, cytochrome c, and β-actin (1:500, Santa Cruz or Abcam) overnight at 4°C and washed 4 times, for 5 minutes each, with TBST. Subsequently, the blots were probed with secondary antibody against rabbit (1:5000, Santa Cruz) conjugated to horseradish peroxidase for 60 min. Following three 5 min washes in PBST, ECL administered on the membranes and radiographic film was used for visualization. For quantification of different proteins, the density values were measured with ImageJ 1.62 software (National Institutes of Health, USA) and normalized to β-actin protein levels.

Statistical analysis
Results obtained were represented as mean ± standard error of the mean (SEM) and analyzed by Graphpad Prism 7.0 software, USA. Statistical comparisons were done by one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test. Values of p < 0.05 was taken as significant.

Results

Levels of Inflammatory Proteins
No significant difference was observed in NF-κβ level between groups (p > 0.05). There were significant differences in protein expressions of TNFα (F (2, 12) = 66.95, p < 0.0001) and TNFR1 (F (2, 12) = 193.3, p < 0.0001). Post hoc analysis indicated no statistical differences in TNFα and TNFR1 levels between control group and sham-operated group (p > 0.05). Protein levels of TNFα and TNFR1 in rats from the ischemic group were significantly upregulated than in the sham group (p < 0.001) (Figure 1A,B).

Levels of Apoptotic Proteins
The results of one-way ANOVA revealed significant differences in cytochrome c (F (2, 12) = 14.37, p = 0.0007), Bax (F (2, 12) = 15.13, p = 0.0005), Bcl-2 (F (2, 12) = 7.608, p = 0.0073), and cleaved caspase-3/procaspase-3 ratios (F (2, 12) = 168.1, p < 0.0001) among groups. No significant differences in the cytochrome c, Bax, Bcl-2, and caspase-3/procaspase-3 ratios between control animals and sham animals were found (p > 0.05). Post-hoc analysis showed a significant increase in the levels of cytochrome c (p < 0.01), Bax (p < 0.01), and caspase-3/procaspase-3 ratios (p < 0.001) of ischemic group compared to sham animals. While Bcl-2 level was significantly decreased in ischemic group when compared with sham animals (p < 0.05) (Figure 2A,B).
Discussion

This study, for the first time, examined the activation of the inflammatory and apoptotic processes following stereotaxic injection of ET-1 in the acute stage of ischemia. We showed that ET-1 upregulates levels of inflammatory biomarkers, TNF-α and TNFR1 except NF-κB, as well as apoptotic molecules, namely cytochrome c, Bax, Bcl-2, and cleaved caspase-3.

ET-1 is used as a method to produce focal brain ischemia in rodents [13]. ET-1 is a potent vasoconstrictor peptide, which can induce ischemic injuries by a reduction in blood flow. In this model, although reduced blood flow is rapid, reperfusion is slow and may occur over several hours [16,17]. Moreover, ET-1 can directly be applied in the target region of brain tissue [18].

The presence of inflammatory mechanisms in the pathophysiology of acute brain ischemia has been well documented [19]. NF-κB is one of the most important transcription factors which is required for the expression of inflammation-related genes. Animal studies of acute ischemia have reported NF-κB overexpression following ischemic injury [20,21]. On the other hand, it has been shown that elevated expression of NF-κB positively modulates the TNF-α expression levels in ischemic tissue. It seems that NF-κB -induced activation of inflammatory cytokines such as TNF-α could be considered as the major inflammatory reaction mechanisms that facilitate ischemic brain damage [20]. Our data in the present study demonstrated that ET-1-induced ischemia could trigger an inflammatory process, which was shown by increasing the inflammatory cytokine TNF-α in 24 hours after ischemia. However, the NF-κB level was reduced, but this reduction was not significant. Other investigators have shown a reduction in NF-κB activity and an increased expression in TNF-α level in an ischemic model of stroke. Botchkina et al. indicated that the decrease of NF-κB level could play an important role in progression TNF-α induced cytotoxicity in cerebral ischemia [22].

TNF-α interacts with two cognate receptors, TNFR1 and TNFR2. TNFR1 is expressed in most cells and, it seems that it is a key mediator of the cytotoxicity of TNF-α through stimulation of the apoptosis pathway [23]. The upregulation of TNFR1 in acute ischemia has been reported [24]. In our study, ET-1-received rats revealed increasing in the TNFR1 level in brain tissue.

Caspases are types of proteolytic enzymes that are expressed as pro-caspases in body tissues and have essential functions in
the apoptosis pathway [25]. Caspase-3 is one of the crucial executors of apoptosis pathway that its activation is an important hallmark of apoptosis after ischemia. The upregulation of caspase-3 protein in brain tissue has been observed 24 hours after focal ischemia [26,27]. The process that ultimately leads to activation of caspase-3, is mediated by Bcl-2 family proteins. Bcl-2 is localized on the mitochondrial membrane and is known as apoptosis-suppressing factor. Bcl-2 prevents apoptosis by inhibiting the release of cytochrome c and reducing the actions of pro-apoptotic molecules such as Bcl-2-associated X protein (Bax). Bax protein accelerates apoptosis events through increased permeabilization of mitochondrial membrane [11]. Studies of acute cerebral ischemia in rodents have provided evidence of the central role of Bcl-2 family proteins in apoptosis, demonstrating that the dys-regulation of the Bcl-2 family members results in the release of cytochrome c and the activation of downstream caspases, thus exacerbate the ischemic injury [28,29]. In the present study, we demonstrated a decrease in the expression of Bcl-2 protein and an increase in the expression of cytochrome c, Bax and, cleaved caspase-3 to procaspase-3 ratio.

**Conclusion**

In conclusion, our results indicated that ET-1 in the acute phase of ischemic injury could trigger inflammatory responses and apoptotic mechanisms.

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**Conflicts of Interest**

The authors declare no conflict of interest.

**References**


