Indian J Physiol Pharmacol 2006; 50 (2): 157–162

PROTECTIVE EFFECT OF ENDOTHELIN ANTAGONIST (TAK-044) ON NEURONAL CELL VIABILITY IN *IN VITRO* OXYGEN-GLUCOSE DEPRIVATION MODEL OF STROKE

SEEMA BRIYAL[1], ADITYA B. PANT[2] AND Y. K. GUPTA[1]*

[1]*Neuropharmacology Laboratory, Department of Pharmacology, All India Institute of Medical Sciences, New Delhi – 575 001*

and

[2]*Division of Developmental Toxicology, Industrial Toxicology Research Centre, Lucknow, India*

(Received on June 30, 2005)

Abstract : The present study was carried to investigate the effect of endothelin antagonist (TAK-044) in an *in vitro* model of stroke using primary neuronal culture. Hypoxia in neuronal culture was induced for 3 h using oxygen glucose deprivation (OGD) model, thereafter cells were reperfused. In separate group cultures were incubated with graded concentrations of TAK-044 (0.01, 0.1 and 1 µg/µl) for different time duration i.e. 6, 12 and 24 hours after reperfusion. Percent cell viability was assessed 24 h after reperfusion using MTT assay.

It was observed that percent cell viability was reduced to 13.7±0.4 % in the cells under 3 h hypoxic condition as compared to the cells under normal condition (100%). TAK-044 at the concentrations of 0.1 and 1 µg/µl, but not at 0.01 µg/µl showed significant ($P<0.01$) improvement in the % cell viability as compared to the cells in hypoxic condition. Percent cell viability at the concentration of 0.1 and 1 µg/µl for 24 h time duration after reperfusion were 54.8±3.2% and 75.4±1.8% respectively as compared to the cells under hypoxic condition (13.7±0.4%). The results demonstrate the neuroprotective effect of TAK-044 against neuronal damage caused by hypoxia induced in neuronal culture.

Key words : ischemic stroke endothelin antagonist TAK-044 neuronal culture neuroprotection

INTRODUCTION

Stroke is a major cause of death and disability and the resulting burden on the society continues to grow, with increase in its incidence (1). Ischemic stroke accounts

*Corresponding Author : Prof. Y. K. Gupta, Department of Pharmacology, All India Medical Sciences, Ansari Nagar, New Delhi – 110 029, India; Tel.: 91-011-26593684, Fax: 91-011-26588641; E-mail : yk.ykgupta@gmail.com

for 80–85% where as hemorrhagic stroke accounts for 15–20% (2). Eighty percent of ischemic stroke occur in the territory of the anterior circulation affecting the territory of the middle cerebral artery (3).

A number of multiple independent lethal mechanisms contribute to the brain injury following the onset of stroke (4, 5). These includes free radical production, excitotoxicity, disruption of sodium and calcium influx, enzymatic changes, stimulation of the inflammatory process, activation of platelets and leukocytes, delayed coagulation, endothelial dysfunction and endothelin (ET) release. Ischemic stroke is characterized by the acute disruption of cerebral blood flow (CBF). The reduction of CBF results in energy failure and secondary biochemical disturbances, eliciting a robust in situ inflammatory response (6). Except tissue-type plasminogen activator (t-PA), no effective therapy exists for the management of acute stroke. Understanding the role of various extrinsic and intrinsic pathogenic factors of ischemic damage particularly the biochemical cascade and neurological changes remain unclear and represent a prime objective of ongoing stroke research (7).

The brain requires glucose and oxygen to maintain neuronal metabolism and function. Hypoxia refers to inadequate delivery of oxygen to the brain, and ischemia results from insufficient CBF. Transient hypoxia-ischemia (HI) leads to delayed neuronal death in both mature and immature neurons (8). Endothelin, a potent vasoconstrictor acts through two receptor subtypes termed as ET (A) and ET (B) (9, 10). It has been reported that ET-1 has a potent contractile effect on cerebral arteries and arterioles (11, 12) causing profound and durable reduction in cerebral blood flow to cause cerebral infarction (13). Increased plasma endothelin levels have been correlated with the infarct size and neurological deficits resulting after focal ischemia (14). ET receptor antagonists are known to exert powerful vasodilatory effect and thereby producing beneficial effect in models of cerebral ischemia. In addition to its vasodilatory effect ET antagonists also have recently been shown to possess an antioxidant (15) and an anti-inflammatory property (16, 17, 18).

TAK-044 (cyclo[D-alpha-aspartyl-3-[(4-phenylpiperazin-1-yl)carbonyl]L-alanyl-L-alphaaspartyl-D-2-(2-thienyl)glycyl-L-leucyl-D-tryptophyl] disodium salt is an ETA- and ETB-receptor antagonist and is able to inhibit ET-1 induced pressor response in the dose range of 1 to 10 mg/kg (19). TAK-044 (1 mg/kg) has shown to have strong inhibitory effects on the extension of myocardial infarct size after coronary artery occlusion-reperfusion in rats (20). Recently we have shown the protective effect of TAK-044 in a model of acute focal cerebral ischemia in rats (21).

Therefore, in the present study graded concentration of endothelin antagonist (TAK-044) was assessed against hypoxia-ischemia in vitro by oxygen-glucose deprivation (OGD) model using neuronal culture.

MATERIAL AND METHODS

Animals

One-day pups were used for the study.

All experimental procedures in rats described were reviewed and approved by the Institutional Animal Ethics Committee.

Drugs and experimental protocol

The concentration of endothelin antagonist TAK-044 (Courtesy Takeda Chemical Industries, Ltd, Osaka, Japan) used were 0.01, 0.1 and 1 µg/µl. TAK-044 was dissolved in normal saline and was made to final volume by mixing an appropriate quantity of culture media for the strength 1 mg/ml. Cultures were incubated with TAK-044 (0.01, 0.1 and 1 µg/µl) for different time duration i.e. 6, 12 and 24 h after reperfusion of the hypoxic cells in separate groups. In all groups percent cell viability was assessed 24 h after reperfusion of the cells using MTT assay.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide) was purchased from Sigma-Aldrich, St. Louis, USA. All culture media were GIBCO/BRL products.

Primary neuronal cell culture

Primary neuronal cultures were prepared according to the method of Yu et al., 1986 (22) with slight modification. In brief, brains from one-day pup were dissected and cerebral hemispheres were removed aseptically and washed in Hank's balance salt solution (HBSS) containing 0.4% gentamycin. Next, the tissue was dissociated by passing through a fire-polished Pasteur pipette and the resultant cell suspension was separated by centrifugation. The pellet then was resuspended in high glucose, Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units of penicillin and 100 µg/ml streptomycin. Cells were counted using hemocytometer and were seeded onto poly-L-lysine coated 96 well plates (10,000 cells per well). The plates were incubated in a CO_2 incubator at 37°C for the development of culture. The medium was replaced with fresh medium twice weekly. Neuronal purity was confirmed with antibodies to neuron-specific enolase (NSE) and more than 95% of all cells in the cultures were immunoreactive for NSE.

Oxygen-glucose deprivation

To model hypoxia-ischemia, cultures were exposed to the oxygen-glucose deprivation condition (23) with some modification. DMEM medium was replaced with glucose free Earle's balanced salt solution. Glucose-deprived cultures were placed in anaerobic chamber (Billups-Rothenberg, Del Mar, CA), which was flushed with 95% N_2 and 5% CO_2 for 3 h. Cells were reperfuse by washed back into glucose-containing DMEM and returned to the normoxic incubator (5% CO_2 and 95% O_2).

Cell viability assessment using MTT assay

Cell viability was monitored by the colorimetric MTT assay as described by Mosmann, 1983 (24). Tetrazolium bromide salt solution was added to cells cultured in microplates. The cells were incubated for 4 h at 37°C and mitochondria dehydrogenase was converted into a colored, water-insoluble formazan salt by the metabolic activity of viable cells. The insoluble formazan is solubilized by adding the DMSO and quantified by Multi-Detection Microplate Reader from BIO-TEK, U.S.A at 530 nm.

Results are expressed as the percentage of viable cells.

Statistical analysis

The results are expressed as mean ± SEM. Statistical analysis for the percent cell viability was conducted using two-way analysis of variance (ANOVA). *P<0.01 represents level of significance.

RESULTS

Effect of graded concentration of endothelin antagonist (TAK-044) on percent cell viability using MTT assay

After 3 h hypoxia the neuronal cells were incubated with graded TAK-044 for different time duration (6, 12 and 24 h). The separate neuronal culture plates were incubated for 6, 12 and 24 h with graded concentration of TAK-044 (0.01, 0.1 and 1 µg/µl). The lowest concentration of TAK-044 (0.01 µg/µl) did not have any effect when incubated for 6, 12 or even 24 h. When the concentration was increased to 0.1 µg/µl, the significant improvement in percent cell viability was observed at 6, 12 and 24 h (24.2±0.9%, 30±0.48% and 54.8±3.2% respectively) (P<0.01). The high concentration of TAK-044 (1 µg/µl) showed dose dependent increase in percent cell viability on incubation for 6, 12 and 24 h (30.3±0.6%, 45.8±2.4% and 75.4±1.8% respectively) as compared to the cells under hypoxic condition (13.7±0.4%) (P<0.01) (Table I).

TABLE I: Effect of endothelin antagonist (TAK-044) on percent cell viability in neuronal culture after inducing 3 h hypoxia using MTT assay. *P<0.01 Vs cells under normal condition; **P<0.01 Vs cells under hypoxic condition.

Groups	*% cell viability*		
Under normal condition	100		
After 3 h hypoxic condition	13.7±0.4*		
	% cell viability 24 h after reperfusion in presence of TAK-044		
Duration of treatment after Hypoxia	6 h	12 h	24 h
Hypoxia-ischemia + TAK-044 (0.1 µg/µl)	24.4±0.9**	30±0.48**	54.8±3.2**
Hypoxia-ischemia + TAK-044 (1 µg/µl)	30.3±0.6**	45.8±2.4**	75.4±1.8**

DISCUSSION

Stroke is one of the foremost causes of morbidity and mortality, and poses a major socioeconomic problem in young patients, especially in developing countries. Despite extensive research, adequate therapies are still elusive. Neuronal degeneration and death are hallmarks of stroke/ischemia (25). Ischemic stroke results from a transient or permanent reduction in cerebral blood flow that is restricted to the territory of a major brain artery. Stroke is the second largest cause of mortality worldwide and is surpassesed only by that of heart disease (26). Brain attack is a term increasingly being used to describe the acute presentation of stroke, and to emphasize the need for urgent remedy (27).

The interruption of blood supply to the brain (ischemia) deprives brain cells of glucose and oxygen, causing irreversible brain damage within minutes. The brain is particularly vulnerable to ischemia because 1) the very high rate of oxidative metabolism, requiring a continuous supply of oxygen and glucose, 2) the metabolic

interdependence of neurons and 3) the sensitivity of neurons to disruption of ion homeostasis brought on by ischemia (28). Cells in ischemic brain tissue undergo a number of changes i.e. free radical production, excitotoxicity, disruption of sodium and calcium influx, enzymatic changes, stimulation of the inflammatory process, activation of platelets and leukocytes, delayed coagulation, endothelial dysfunction and endothelin (ET) release (4, 5).

The OGD cell culture lesion model represents a valid simulation of the conditions in brain ischemia. For this cell cultures are maintained under oxygen free conditions in a hypoxic chamber and subjected to glucose deprivation. Therefore in the present study, OGD was selected for the evaluation of endothelin antagonist (TAK-044) in brain ischemia.

Results have demonstrated that neuronal cells under hypoxic condition showed reduction in percent cell viability as compared to cells under normal condition. When the hypoxic neuronal culture was incubated with TAK-044 at different concentration for different time duration in the separate groups. The increase in viability of neuronal cells in the presences of TAK-044 demonstrates the neuroprotective effect of endothelin antagonist. As the neuronal viability was improved after the 3 h of hypoxic injury, such compounds warrant further study to demonstrate if they can be useful in reducing the effect of ischemic insult.

The present study suggests the potential pharmacological intervention of endothelin antagonist (TAK-044) in acute ischemic stroke.

ACKNOWLEDGMENTS

Endothelin antagonist (TAK-044) was a generous gift from Dr. Sonoko Fujiwara, Takeda Chemical Industries, Ltd, Osaka, Japan through Dr. Anil Gulati, department of Biopharmaceutical Sciences (M/C 865), University of Illinois at Chicago, Chicago, IL 60612, USA. Authors would like to thanks Indian Council of Medical Research (ICMR), New Delhi for the funding support.

REFERENCES

1. Kaste M. Thrombolysis in ischaemic stroke - present and future: role of combined therapy. *Cerebrovascular Diseases* 2001; 11(1): 55–59.
2. Gorelick PB. Cerebrovascular disease. Pathophysiology and diagnosis. *Nurs Clin North Am* 1986; 21(2): 275–288.
3. Sudlow CLM, Warlow CP. Comparable studies of the incidence of stroke and its pathological types. *Stroke* 1997; 28: 491–499.
4. Scott JF, Gray CS. Cerebral and systemic pathophysiological responses to acute stroke. *Age and Ageing* 2000; 29: 197–202.
5. Cheung RT. The utility of melatonin in reducing cerebral damage resulting from ischemia and reperfusion. *J Pineal Res* 2003; 34: 153–160.
6. Zheng Z, Yenari MA. Post-ischemic inflammation: molecular mechanisms and therapeutic implications. *Neurol Res* 2004 Dec; 26(8): 884–892.
7. Aronowski J, Labiche LA. Perspectives on reperfusion-induced damage in rodent models of experimental focal ischemia and role of gamma-protein kinase C. *ILAR J* 2003; 44: 105–119.
8. Liu CL, Siesjo BK, Hu BR. Pathogenesis of hippocampal neuronal death after hypoxia-

ischemia changes during brain development. *Neuroscience* 2004; 127(1): 113–123.

9. Wu-Wong JR, Chiou WH, Huang ZJ, Vidal MJ, Opgenorth TJ. Endothelin receptors in human smooth muscle cells: antagonist potency differs on agonist-evoked responses. *American Journal of Physiology-Cell Physiology* 1994; 267: C1185–C1195.

10. Wu-Wong JR, Dayton BD, Opgenorth TJ. Endothelin-1-evoked arachidonic acid release: a Ca^{2+}-dependent pathway. *American Journal of Physiology-Cell Physiology* 1996; 271: C869–C877.

11. Lee ME, de la Monte SM, Ng SC, Bloch KD, Quertermous T. Expression of the potent vasoconstrictor endothelin in the human central nervous system. *Journal of Clinical Investigation* 1990; 86: 141–147.

12. Viossat I, Duverer D, Chapelat M, Pirotzky E, Chabrier PE, Braquet P. Elevated tissue endothelin content during focal cerebral ischemia in the rat. *Journal of Cardiovascular Pharmacology* 1993; 22: S306–S309.

13. Robinson MJ, Macrae IM, Todd M, Reid JL, McCulloch J. Reduction of local cerebral blood flow to pathological levels by endothelin-1 applied to middle cerebral artery in the rat. *Neuroscience Letters* 1990; 118: 269–272.

14. Estrada V, Tellez MJ, Moya J, Fernandez-Durango R, Egido J, Fernandez Cruz AF. High plasma levels of endothelin-1 and atrial natriuretic peptide in patients with acute ischemic stroke. *American Journal of Hypertension* 1994; 7(12): 1085–1089.

15. Andrasi TB, Kekesi V, Blazovics A, Dobi I, Szabo G, Juhasz-Nagy A. ET(A) receptor blockade protects the small intestine against ischaemia/reperfusion injury in dogs via an enhancement of antioxidant defences. *Clinical Science (London)* 2002; 148: 59S–63S.

16. Bajory Z, Hutter J, Krombach F, Messmer K. The role of endothelin-1 in ischemia-reperfusion induced acute inflammation of the bladder in rats. *Journal of Urology* 2002; 168: 1222–1225.

17. Lehmberg J, Putz C, Furst M, Beck J, Baethmann A, Uhl E. Impact of the endothelin-A receptor antagonist BQ 610 on microcirculation in global cerebral ischemia and reperfusion. *Brain Research* 2003; 961: 277–286.

18. Baamonde A, Lastra A, Villazon M, Bordallo J, Hidalgo A, Menendez L. Involvement of endogenous endothelins in thermal and mechanical inflammatory hyperalgesia in mice. Naunyn-Schmiedeberg's Archives of Pharmacology 2004; 369: 245–251.

19. Ikeda S, Awane Y, Kusumoto K, Wakimasu M, Watanabe T, Fujino M. A new endothelin receptor antagonist, TAK-044, shows long-lasting inhibition of both ETA- and ETB-mediated blood pressure responses in rats. *Journal of Pharmacology and Experimental Therapeutics* 1994; 270(2); 728–733.

20. Watanabe T, Awane Y, Ikeda S, Fujiwara S, Kubo K, Kikuchi T, Kusumoto K, Wakimasu M, Fujino M. Pharmacology of a non-selective ETA and ETB receptor antagonist, TAK-044 and the inhibition of myocardial infarct size in rats. *British Journal of Pharmacology* 1995; 114(5): 949–954.

21. Gupta YK, Briyal S, Sharma U, Jagannathan NR, Gulati A. Effect of endothelin antagonist (TAK-044) on cerebral ischemic volume, oxidative stress markers and neurobehavioral parameters in the middle cerebral artery occlusion model of stroke in rats. *Life Sci* 2005 May 20; 77(1): 15–27.

22. Yu AC, Chan PH, Fishman RA. Effects of arachidonic acid on glutamate and gamma-aminobutyric acid uptake in primary cultures of rat cerebral cortical astrocytes and neurons. *J Neurochem* 1986 Oct; 47(4): 1181–1189.

23. Koretz B, von B Ahern K, Wang N, Lustig HS, Greenberg DA. Pre-and post-synaptic modulators of excitatory neurotransmission: comparative effects on hypoxia/hypoglycemia in cortical cultures. *Brain Res* 1994 Apr 18; 643(1–2): 334–337.

24. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983; 65: 55–63.

25. Prunell GF, Arboleda VA, Troy CM. Caspase function in neuronal death: delineation of the role of caspases in ischemia. *Curr Drug Targets CNS Neurol Disord* 2005 Feb; 4(1): 51–61.

26. Bonita R. Stroke prevention: a global perspective. In: Norris JW. Hachinski V. eds. Stroke prevention. New York. NY: Oxford University Press 2001: 259–274.

27. Gupta YK, Briyal S. Animal models of cerebral ischemia for evaluation of drugs. *Indian J Physiol Pharmac* 2004; 48(4): 379–394.

28. Gupta YK, Sharma M. Oxidative stress in neurological disorders. Toxicology and Environmental Health. Eds SB Vohora and VP Agrawal. Asiatech Publ. Inc., New Delhi. 1999. pp 65–77.