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Alterations of Inducible and Constitutive Nitric Oxide Synthase after Hippocampal Injury in Rats

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Abstract: The aim of this study was to study the changes of inducible and constitutive Nitric Oxide Synthase (NOS) after brain injury. In order to brain injury 42 wistar rats were submitted and divided in 7 groups. Nitric oxide synthase activities were assayed at different times after injury. Present results showed that a significant increase of iNOS and cNOS activity 8 h after lesion. In conclusion, both isoforms of NOS increase at different time after brain injury.

Key words: Brain injury, nitric oxide, synthase, inducible, constitutive

INTRODUCTION

Traumatic Brain Injury (TBI) induces a complex cascade of pathophysiological changes that spread from primary lesion to the adjacent tissues (Ziaja *et al.*, 2007). Some process in CNS damage includes, ischemia, inflammatory responses, alteration of energetic metabolism. As a result some important amino acids such as glutamate, aspartate are altered after damage and this lead to dysregulation of some metabolic pathway (Diaz-Ruiz *et al.*, 2007). Among the important free radicals that produced during brain damage is Nitric Oxide (NO). NO is synthesized from L-arginin by two classes of enzymes termed constitutive nitric oxide Synthase (cNOS) and inducible (iNOS), the former dependent on intra cellular calcium and the latter independent. In the CNS two different cNOS isoforms are present, one in neurons (nNOS) and in endothelial cells (eNOS) (Hierholzer *et al.*, 1998; Martinez-Murillo *et al.*, 2007). CNS and neural cells have a great Ca²⁺/calmodulin regulated NOS activity and NO is an important biological messenger in the CNS (Matsumoto *et al.*, 1993). NOS have been purified from microglia (Dinerman *et al.*, 1994), cerebellum, hippocampus (Gham *et al.*, 2002), striatum (Sardo *et al.*, 2002). It has been suggested that NO may play a role in learning, memory and development of the brain (Delwing *et al.*, 2008). There is a little information about NOS activity after brain injury (Diaz-Ruiz *et al.*, 2002). Diazal-Ruiz *et al.* (2002) showed a significant increase in cNOS activity at 4 and 8 h and iNOS activity was increased 72 h after spinal cord injury (Hu *et al.*, 2008). Hu *et al.* (2008) showed after spinal cord injury a time-

dependent increase in cNOS and nNOS activity. Xu *et al.* (2000) showed after traumatic injury of CNS the activity of iNOS increased. Cherian *et al.* (2000) reported that after brain injury in rats pretreated with L arginin concentration of NO were increased. We hypothesized that enhanced NO production resulting from iNOS and eNOS expression would contribute to inflammatory signaling in brain damage. In the present study, we inflicted a contusion to the brain cause a changes of cNOS and iNOS activities in rats.

MATERIALS AND METHODS

Animal: The animal experiments were conducted in Pharmacology and Anatomy Department and NOS measurement works were conducted in cellular and molecular research center of Iran medical university from 2006-2008.

Adult male Wistar strain of rats (about 3 months of age) were used and were housed in a cages with a 12 h light/dark cycle at 22-24°C temperature, with food and water *adlibitum*. All experimental procedures were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals and all experiments conformed to Iran University of medical sciences guidelines on the ethical use of laboratory animals. Also, in each experiment care was taken to minimize the animals suffering and to use the minimum number of animals.

Hippocampus injury: The rats were randomly allocated in two groups. One group (lesion group n = 6 in each

subgroup) was submitted to a sever hippocampal injury according with the injection of a canola in to the right hippocampus with the stereotaxic apparatus (3.8 mm from bregma, 2.6 mm from the mid line and 3.7 mm vertically) according to atlas of the rat brain (Paxinos and Watson, 2005). This coordination has been used effectively in several studies for the hippocampus. The second group was only submitted to the drain of skull (sham group n = 6) under pentobarbital anesthesia.

Measurement of NO: NOS activity were assayed by measuring the conversion of [³H] - L-arginin to the [³H] - L-Citrulline (Bredt and Synder, 1990). Both groups allowed to recover from anesthesia and surgical procedures, then housed in individual cages. Rats take food and water *ad libitum*.

Animal were killed at different time after surgery (2, 4, 6, 8, 12, 24 and 72 h). After decapitation right hippocampus were removed and then immersed in liquid nitrogen and stored at -70°C until ready for NOS activity measurement. For inhibition of enzyme protease all separated hippocampus (in a group) homogenized for 15 min in a solution that containing (50 mM Tris-Hcl, 0.1 mM EDTA, 0.1% 2-me, 1 mM leupeptin, 1 mM PMSF, 2 mg L⁻¹ aprotinin. A volume of sample containing 500 µg of protein was incubated in the presence of 10 µM L-arginin, 0.2 µCi [³H] L-arginin, 1 mM NADPH, 10 µM calmoduline and 2 mM CaCl₂. The reaction was stopped by addition of 1 mL stopping buffer that contain (3.5 mM EDTA, 20 mM HEPES). The mixture was then runs onto a resin column that equilibrated with stop buffer Results Were expressed as ng of L-Citrulline/mg protein/30 min. Because c NOS have calcium dependent activity and iNOS have calcium-independent activity the enzyme activity were evaluated either in the presence of 2.5 mM and the absence of calcium in incubation medium (Diaz-Ruiz *et al.*, 2007). Protein concentration was measured by the Lowry method (Lowry *et al.*, 1954).

Statistical analysis: The data obtained from counting of protein concentrations. And analyzed by using SPSS version 12. Then variables have been analyzed by one-way Analysis of Variance (ANOVA) followed by Tukey post hoc test. All data were expressed as the Mean±SD and the differences were considered to be significant when p<0.05.

RESULTS

Results of NOS activities in Fig. 1 and 2 have shown that in the control groups hippocampus have cNOS (17.92±0/7) and iNOS (13.61±0/64) basal activities. Both

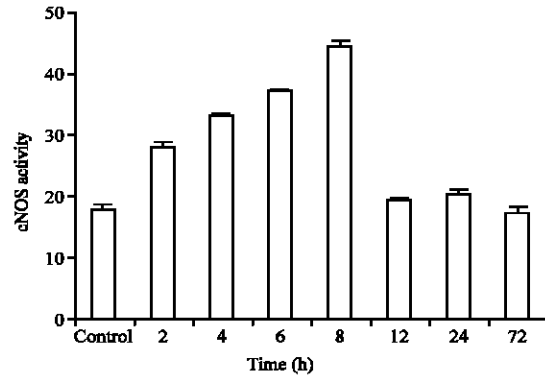


Fig. 1: Constitutive nitric oxide synthase (cNOS) activity measured in several time after brain injury (2, 4, 6, 8, 12, 24 and 72 h). Control group received only skull drain and was scarified 2 h after injury. The results are expressed as Mean±SD (p<0.05)

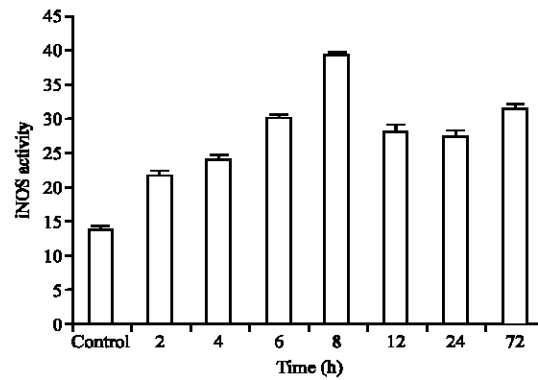


Fig. 2: Inducible nitric oxide synthase (iNOS) activity measured in several time after brain injury (2, 4, 6, 8, 12, 24 and 72 h). Control group received only skull drain and was scarified 2 h after injury. The results are expressed as Mean±SD (p<0.05)

enzymes presented a trend to increase their activity in injured animals. cNOS activity was increased (Fig. 1) in the injured animal after 2 h (28.19±0/58) (157%) and gradually increased to maximum concentration at 8 h (44.86±0/66) (250%) after injury and then slowly reduced to at 72 h (17.23±0/93). In the injured animals in the groups of rats were killed at 12, 24 and 72 h are near or equal to control levels. iNOS activities in the brain of control rats was (13.61±0/64). Interestingly, this enzyme gradually increased in injured animals. After 8 h that was maximum concentration (39.11±0/50) (287%) and then slowly decreased to 72 h after injury (31.3±0/73) (230%) but are still well above control levels (Fig. 1, 2). cNOS activity was expressed

higher than iNOS activity, but iNOS activity was expressed at between 8 to 72 h more significantly than cNOS activity.

Concentration of cNOS activity compared to iNOS at 8 h post injury was significant.

DISCUSSION

In the present study, the activity of both isoforms of NOS was measured at different period of time in brain injury. Results indicate a time dependent sequential increase activity in both cNOS and iNOS enzyme after injury.

Present results are agreement with data of Diaz-Ruiz *et al.* (2002) and Hu *et al.* (2008) who showed activity of cNOS increased within the first 4 h after spinal cord injury and then gradually decreased to 72 h and the maximum concentration was in 4 h after surgery, but the present data show activity of cNOS increased within the first 8 h after injury differences in the site of damage could be responsible for these versatilities.

cNOS activity after 4 h was 138% (Cherian *et al.*, 2000) but in this study the percent of cNOS increased to 250% after 8 h and this data is considerable of course the base of cNOS in the brain is higher than spinal cord and since NO have a neuroprotective effects as a vasodilatation and neuronal function the injury of the brain cause a significant increase of NO in the brain.

Injury of the brain leads to a strong up regulation of iNOS production in a time dependent manner so cause increase iNOS protein level in injured site. According to study of Huttemann *et al.* (2008) the maximal level of iNOS was 4 h after injury then decreased to 48 h. But the peak activity of iNOS in our result was 8 h and then reduced. Induction of brain injury that used by Huttemann *et al.* (2008) cause damage to expanded area of brain and this wide damage cause earlier pick activity of iNOS.

Increased cNOS activity at short time 8 h is perhaps related to calcium dependent mechanisms evoked by the tissue response to damage (Liu *et al.*, 1991). And Increase of iNOS activity is subsequent inflammatory response (Clark *et al.*, 1996) and macrophages recruitment after damage (Clark *et al.*, 1996) because the peak activity of iNOS was lower than cNOS level. Present results showed an earlier pick activity in both enzymes.

The increase in NO is subsequent higher concentration of nitrate/nitrite during the first 20 min after injury (Hu *et al.*, 2008). Sakomoto (1997) demonstrate a nearly 2.5 fold increase in the concentration of NO in 10 min after brain injury. The mechanism of the initial increase in NO isn't completely understood. Extraordinary metabolic changes occur in the time of brain injury and

after that. At the time of injury sever depolarization occurs in the injury site and around cells, potassium concentration in extracellular space and calcium in intracellular increase (Yamada and Nabeshima, 1997). The brain glucose increase Glutamate release. So, increase of NO based on these results is that glutamate stimulate the NMDA receptors and then causes influx of post synaptic calcium and therefore activation of the constitutive isoforms of NO.

Some research show concentration of NO reduces immediately after brain injury. The mechanism of reduce is not well understood in the present study cNOS increase 2 h after injury and then reduced 12 h post injury. Local depletion of substrate for NOS may play a role in reducing NO production (Leone *et al.*, 1991). Polyamines ornithine decarboxylase elevated after trauma (Henley *et al.*, 1996). The effect of this amine is cytotoxic. Polyamines are toxic to granule cells (Sparapani *et al.*, 1997). So, effects by inhibiting NOS or by blocking inward rectifier K⁺ channels (Ficker *et al.*, 1994). Free radicals such as superoxide produce after injury and may be inactivated NO and this is a mechanism for reduction of NO after 12 h post injury (Dewitt *et al.*, 1997).

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