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Original Article

Effects of preconditioning with intermittent normobaric hyperoxia on TNFR1 and TNFR2 expression in the rat brain

Firoozeh Alavian[1], Sohrab Hajizadeh[2]*, Mohammad Javan[2], Mohammad Reza Bigdeli[3]

1. Farhangian University, Tehran, Iran
2. Department of Physiology, School of Medical Sciences, Tarbiat Modares University, Tehran, Iran
3. Department of Biology, Shahid Beheshti University, Tehran, Iran

Abstract

Introduction: Recent studies have shown that intermittent normobaric hyperoxia (HO) protects the rat brain from ischemia reperfusion injury. However, the exact mechanism of this kind of protection *in vivo* is not known. In this study, the effect of HO on expression of TNFR1 and TNFR2 in a stroke model was investigated.

Methods: In this experimental study, rats were divided into 4 groups: normoxia – sham, hyperoxia – sham, normoxia – stroke and hyperoxia –stroke for each factor (TNFR1 or TNFR2). Hyperoxia groups were exposed to 95% inspired oxygen for 4 h/day and 6 consecutive days. Oxygen concentration in the control groups was 21% (normoxia, room air). After 24h, the rats were subjected to 60 min of right middle cerebral artery occlusion (MCAO). After 24h reperfusion, neurological deficit scores (NDS) and TNFR1, 2 brain levels using Western Blot were assessed.

Results: Preconditioning with HO decreased NDS. Also, followed by stroke and reperfusion, TNFR1 levels significantly increased; while there was no significant difference in hyperoxia groups compared with normoxia groups in the cortex, HO significantly reduced TNFR1 expression in subcortex. On the other hand, groups of stroke compared to sham groups significantly expressed lower levels of TNFR2 in the cortex and subcortex. There was no significant difference in hyperoxia groups compared with normoxia groups in these areas.

Conclusion: Although additional studies will be required to further elucidate precise mechanisms of ischemic tolerance, it seems that HO is associated with the expression of TNFR1 in subcortex, consistent with an active role in the genesis of ischemic protection.

Keywords:
Hyperoxia;
Stroke;
MCAO;
TNFR1;
TNFR2

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***Correspondence to:**
S. Hajizadeh

Tel: +98-2182883523
Fax: +98-2182884555

Email:
hajizads@modares.ac.ir

Introduction

Currently, stroke is the fourth leading cause of death in the United States and a leading cause of disability (Ovbiagele et al., 2013). Ischemic preconditioning (IPC) is an endogenous phenomenon that whereby concise periods of ischemia, causes a tissue more resistant to subsequent sustained loss of blood flow and can cause ischemic tolerance (IT) in organs such as the brain and heart (Kitagawa et al., 1990). IT involved in the adjustment mechanism such as the

synthesis of anti-oxidant enzymes, new protective proteins, growth factors or anti-apoptotic gene products (Chen and Simon 1997; Kato et al., 1992; Kirino, 2002; Ohtsuki et al., 1992; Perez-Pinzon et al., 1996). Normobaric hyperoxia (HO) is one of the safe nonpharmacologic stimuli which maybe through the generation of oxygen free radicals and hydroxyl radicals protects the rat brain tissue from ischemia reperfusion injury (Bigdeli et al., 2007; Wada et al., 2000). The exact mechanism of this effect is not well understood but it appears that enhancement of endogenous defense mechanisms are involved.

The maintenance of cerebral IT may be mediated through changes in expression a variety of mediators, including tumor necrosis factor-α (TNFα), TNFα converting enzyme (TACE), hypoxia-inducible factor 1α (HIF1α), mitochondrial uncoupling protein 2 and sodium-calcium exchanger 1 (Alavian et al., 2012a; Alavian et al., 2012b; Bigdeli et al., 2009; Mohammadi and Bigdeli, 2014). It is possible that TNFα can be an important mediator of IT (Liu et al., 2000). TNF was detected in 1975 as an endotoxin-inducible molecule that caused necrosis of tumors *in vitro* (Carswell et al., 1975). TNF is a transmembrane 26 KDa protein which often expressed by monocytes, macrophages, activated T cells and microglia of central nervous system (CNS) (Sedger and McDermott, 2014). This factor is released in soluble form by the metalloprotease TNF alpha converting enzyme which is called TACE. TACE activation might be a common signal transduction factor in brain which initiates different signalling pathways during IPC-induced tolerance (Black et al., 1997).

TNFα is an important effector of inflammatory responses. In various types of inflammation, the local release of TNFα occurs (Tacchini-Cottier et al., 1998), So that inhibition of TNFα, reduces inflammation and pain (Yamacita-Borin et al., 2015). Also, further demonstrated that the actions of TNF were similar to that of the endotoxin (Parameswaran and Patial, 2010).

Recently, role of TNFα in the brain mechanisms of protection against damage which caused by ischemia is greatly emphasized (Shohami et al., 1999). This factor effects through two glycoprotein membrane receptor; TNFR1 (a death-domain-containing protein which known as p55/p60) and TNFR2 (also sometimes referred to a p75/p80) (Beutler and van Huffel, 1994; Sedger and McDermott, 2014). The expression levels of TNFRs can be adjusted by cytokines such as interferons (Aggarwal et al., 1985). While most cells expressed consistently low levels of TNFR1, only some cells such as endothelial cells, immune cells and several types of CNS cells (oligodendrocytes, microglia, astrocytes and some neuronal populations) express detectable levels of TNFR2. Unlike TNFR1, TNFR2 has no death domain (Choi et al., 2005; Sedger and McDermott, 2014). TNFR2 activates nuclear factor-kappa B (NF-κB) and c-Jun N-terminal kinase which leads to transcriptional activation of genes related to cell proliferation and survival. Strong activation of NF-κB increased the nerve cells survival. Also, in absence of NF-κB, sensitivity to TNFα and apoptosis increases (Cabal-Hierro et al., 2014). TNFR1 has the most important role in the biological activity of TNFα; its activation may result in either cell proliferation or cell death.

It was demonstrated that superoxide anion-induced pain and inflammation depends on TNFα/TNFR1 signalling in mice. Also, TNFR1-specific activity was increased in response to reactive oxygen species (ROS) (Yamacita-Borin et al., 2015). So oxygen species can determine activity path of TNFα.

In isolated rat heart cells, TNFα induced ROS production and altered cell survival. By using anti-TNFR2 antibodies, intensified TNFα responses on ROS production and cell death, arguing for an important protective role of the TNFR2 pathway. In total, there are close interaction between TNFR1 and TNFR2 pathways in cardiac myocytes (Defer et al., 2007).

TNFR2 plays a marginal role during retinal vascular evolution. TNFR2 in vascular endothelial cells strongly prevents hyperoxia-induced vaso-obliteration by inhibiting cell apoptosis and promotes retinal repair. Therefore, activation of TNFR2 signalling may be an ideal strategy for the treatment of oxygen-induced retinopathy (Wan et al., 2013).

In spite of above information, the role of TNFR1 and TNFR2 in the induction of the ischemic tolerance by HO in brain is still not well understood. Activation of these two receptors by oxidants, prompted us to investigate the potential role of TNFR1 and TNFR2 under HO in rat brain stroke model.

Materials and methods

Animals and group assignment

Adult male Wistar rats (250-350 g) were housed under conditions of controlled temperature (22±2 °C) and constant humidity with 12 h light/dark cycle (light on 07:00–19:00) for all experiments. All experimental animal procedures were conducted with the approval of the Ethics Committee of the Tarbiat Modares University. Every effort was made to minimize the number of animals used and their suffering.

Rats were divided randomly into 4 groups (n=6 animals in each group). Two groups were placed in an environmental chamber and exposed to a hyperoxic atmosphere (95% oxygen: normobaric hyperoxic groups, or HO) for 4 continuous hours for six consecutive days; of this four groups, two were sham and the other two groups subjected to middle cerebral artery occlusion (MCAO). Two other group were similarly placed in the environmental chamber and exposed to room-air (RA) equivalent (21% oxygen: normobaric normoxic groups) for similar time periods and the same division of HO groups.

Environmental chamber

HO treatment was initiated in the chamber (650×350×450 mm^3). The oxygen concentration inside the container was continuously monitored using an oxygen sensor (Lutron-Do5510, Taiwan) and soda lime; a carbon dioxide absorber was used (BDH Limited, Poole, England) at the sides of the chamber.

Operative procedures

At the beginning of day 7 after induction of HO, animals were anesthetized with chloral hydrate (400 mg/kg) and were subjected to MCAO as described previously (Longa et al., 1989). In brief, the right common carotid artery (CCA) was carefully separated from the vagus nerve and surrounding tissues. Using microscopic surgery, a 3-0 silicone coated nylon suture was introduced through the external carotid artery stump. The occluder was advanced into the internal carotid artery 20–22mm beyond the carotid bifurcation until mild resistance indicated that the tip was lodged in the anterior cerebral artery and blocked the blood flow to the middle cerebral artery. Rectal temperature was monitored (Citizen-513w) and maintained at 37.0 °C by surface heating and cooling during surgery. The body temperature, blood gases and heart rate were maintained within the physiologic range throughout the operation. Reperfusion was started by removing the suture after 1 hour of ischemia. The sham operated rats were subjected to the same surgical procedures except the filament insertion.

The measurements of blood pressure and the number of respiratory movements were performed using an arterial catheter connected to a pressure transducer and a Power Lab device (AD instrument Co, Australia). Also, the respiratory rate was measured by movement's number of animals' abdomen.

Arterial blood gas analysis

Sampling was performed from the carotid artery of the neck at 4 °C and with a curved needle to prevent the entry of air into the needle. To measure blood gas, samples were transferred to Shariati Hospital and used arterial blood gas (ABG) devices.

Laser–Doppler flowmetry

Continuous laser–Doppler flowmetry (LDF; Moor Instrument, UK) was used to monitor cerebral perfusion to ensure adequacy of MCAO through the CCA. Using a stereotaxic apparatus and a low-speed dental drill, a burr hole of 2 mm in diameter was made over the skull at 1 mm posterior and 5 mm lateral to the Bregma on the right side. A needle shaped laser probe was placed on the dura away from visible cerebral vessels (Chen and Cheung, 2002). Doppler flux recording was started 30 minutes before MCAO operation (steady-state baseline values were recorded before MCAO) and continued until the laser-Doppler signal showed a steep decrease (more than 75% reduction of cerebral blood flow is necessary for successful induction of ischemia) (Mohammadi and Bigdeli, 2014).

Neurobehavioral evaluation

After the operation, rats were returned to their separate cages. Twenty four hour after surgery, the rats were assessed neurologically by an observer who was blind to the animal groups. The neurological deficit scores (NDSs) was performed using a six-point scale (Longa et al., 1989): normal motor function=0; failure to extend left forepaw fully=1; circling to the contralateral side but have normal posture at rest=2; falling to the left, a severe focal deficit=3; rats that did not walk spontaneously and had a depressed level of consciousness=4. Death was considered as score 5

only when a large infarct volume was present in the absence of subarachnoid haemorrhage. If the rats died due to subarachnoid haemorrhage or pulmonary insufficiency and asphyxia, they were eliminated from the study.

Brain sampling and protein extraction

After 48 hours of surgery, intact and sham animals were killed by chloral hydrate (800 mg/kg) and decapitated for the measurement of protein expression. For this purpose, the brains were removed and hemispheres separated. Then, cortex and subcortex were carefully separated; considering the boundary between brain's white and gray areas (Mohammadi and Bigdeli, 2014). In the next step, cells of the cortex and subcortex areas were homogenized by sonication at 4 °C in 4 volumes of buffer containing 0.5% sodium deoxy cholate, 150 mM NaCl, 0.1% SDS, 0.03% EDTA, 1 tablet protease inhibitor cocktail (Roche, Switzerland) and 50 mM Tris–HCl with pH 7.0 (homogenization buffer) (Mohammadi and Bigdeli, 2013).

Western blot analysis

Homogenate containing about 60 µg of total protein from the cortex and subcortex of right hemisphere samples (sham-operated group, hyperoxia and RA subgroups) were loaded together with a protein ladder (Thermo Scientific, Deutschland) and the proteins size-separated in 8% SDS–PAGE (sodium dodecyl sulphate–polyacrylamide gelelectrophoresis) (90 mA). The proteins were blotted onto a polyvinylidene fluoride membrane (Millipore). Blots were blocked at room temperature for 1 h in blocking reagent (GE Health Care, United State) and subsequently incubated with specific polyclonal goat antibody to TNFR1 (200 µg/ml, Santa Cruz Biotechnology), polyclonal rabbit antibody to TNFR2 (0.1-0.5 µg/ml, antibody resource) and rabbit antibody against β-actin (1:1000 dilution, Santa Cruz) separately. They were then incubated with secondary anti-rabbit (1:10000 dilution) (Dakocytomation, Denmark) and rabbit anti-goat (1:2000) (ab97100, Abcam). Antibodies conjugated to horseradish peroxidase for 1 h at room temperature. TNFR1 and 2 immune-reactive proteins were detected with advanced chemiluminescence (Enhanced Chemiluminescence, Amersham Biosciences) and film exposure. After scanning and transferring the film images to a computer, signal bands were quantified (Image J software). Densitometry analyses of bands after normalization with β-actin as a loading control were calculated as a present of that seen in the RA group.

Statistical analysis

Data were expressed as means ± SEM. ABG were compared using two-way ANOVA test. The NDSs were analysed using the Bonferoni post-hoc test. $P<0.05$ was considered significant.

Results

Figure 1 shows oxygen content (%) in the container in HO and normoxia conditions. Arterial blood gas analysis confirmed clinical HO in the treated groups (Table 1). MCAO was able to reduce regional cerebral blood flow to less than 24% of that at baseline; the average which is considered for rat exposed to HO and RA (Fig. 2).

Effects of HO on NDS

Hyperoxia decreased NDS and improved the behaviour; so that hyperoxia treated groups had lower neurologic deficit scores, respectively (Table 2).

Effects of HO-induced neuroprotection on TNFR1 expression

As shown in Figure 3, comparison TNFR1 expression at the protein level using two-way ANOVA shows that in cortex (Fig. 3A), groups of stroke compared to sham groups significantly express higher levels of TNFR1 ($F_{stroke\ vs\ sham}=33.56$, $P<0.001$). Hyperoxia group protein levels were not significantly different compared with normoxia groups ($F_{HO\ vs\ RA}=6.203$, $P>0.05$). Well as, interaction between the groups was statistically significant ($F_{interaction}=10.24$, $P<0.05$). In the subcortex areas (Fig. 3B) similar to the cortex, stroke leading to significant increase of TNFR1 protein expression compared to sham groups ($F_{stroke\ vs\ sham}=31.04$, $P<0.01$). In this area, hyperoxia caused a significant reduction in TNFR1 expression compared with normoxia ($F_{HO\ vs\ RA}=10.98$, $P<0.05$). Interaction between hyperoxia and normoxia groups with shams and stroke was significant ($F_{interaction}=19.14$, $P<0.05$).

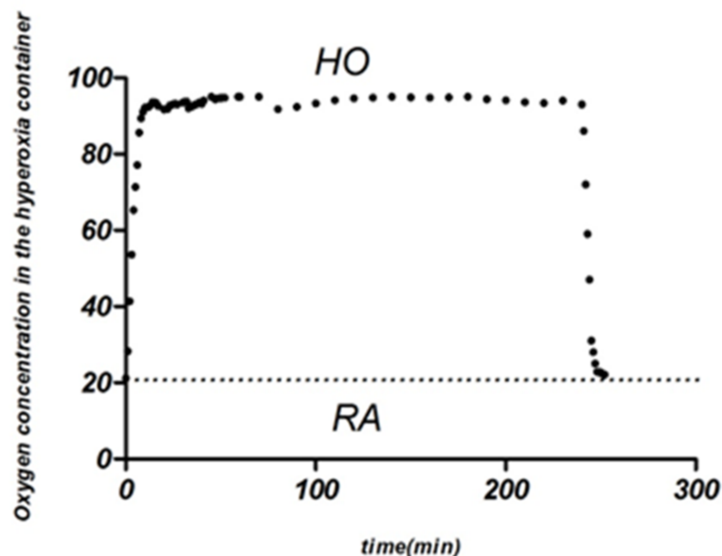

Fig.1. Oxygen concentration in the hyperoxia-inducing chamber during hyperoxia (HO) in comparison with room air (RA)

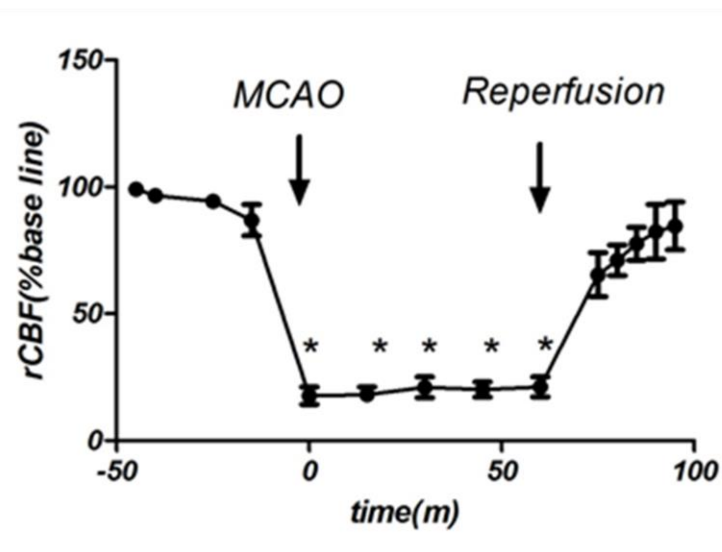

Fig.2. Average regional cerebral blood flow (rCBF) before and during middle cerebral artery occlusion (MCAO) and after reperfusion (n=6, data as mean ± SEM and **P*<0.05).

Effects of HO-induced neuroprotection on TNFR2 expression

Comparison TNFR1 expression at the protein level (Fig. 4) using two-way ANOVA shows that in cortex (Fig. 4A), groups of stroke compared to sham groups significantly express lower levels of TNFR2 (Fstroke vs sham=33.56, P<0.01). Hyperoxia group protein levels were not significantly different compared with normoxia groups (FHO vs RA=6.203, P>0.05). Well as, interaction between the groups was statistically significant (Finteraction=10.24, P<0.05). In the subcortex areas (Fig. 4B) similar to the cortex, stroke leading to significant reduction of TNFR2 protein expression compared to sham groups ($F_{stroke\ vs\ sham}$=183.8, P<0.05). Hyperoxia in this area compared with normoxia had no significant effect on the expression of TNFR2 ($F_{HO\ vs\ RA}$=3.677). Interaction between hyperoxia and normoxia groups with shams and stroke was significant (Finteraction=14.51, P<0.05).

Discussion

In this study effects of hyperoxia on the expression of TNFR1, 2 and neurological defects were investigated. According to our results, it seems that the hyperoxia can reduce NDSs in MCAO model. Previous studies show that intermittent HO which induces IT, plays an

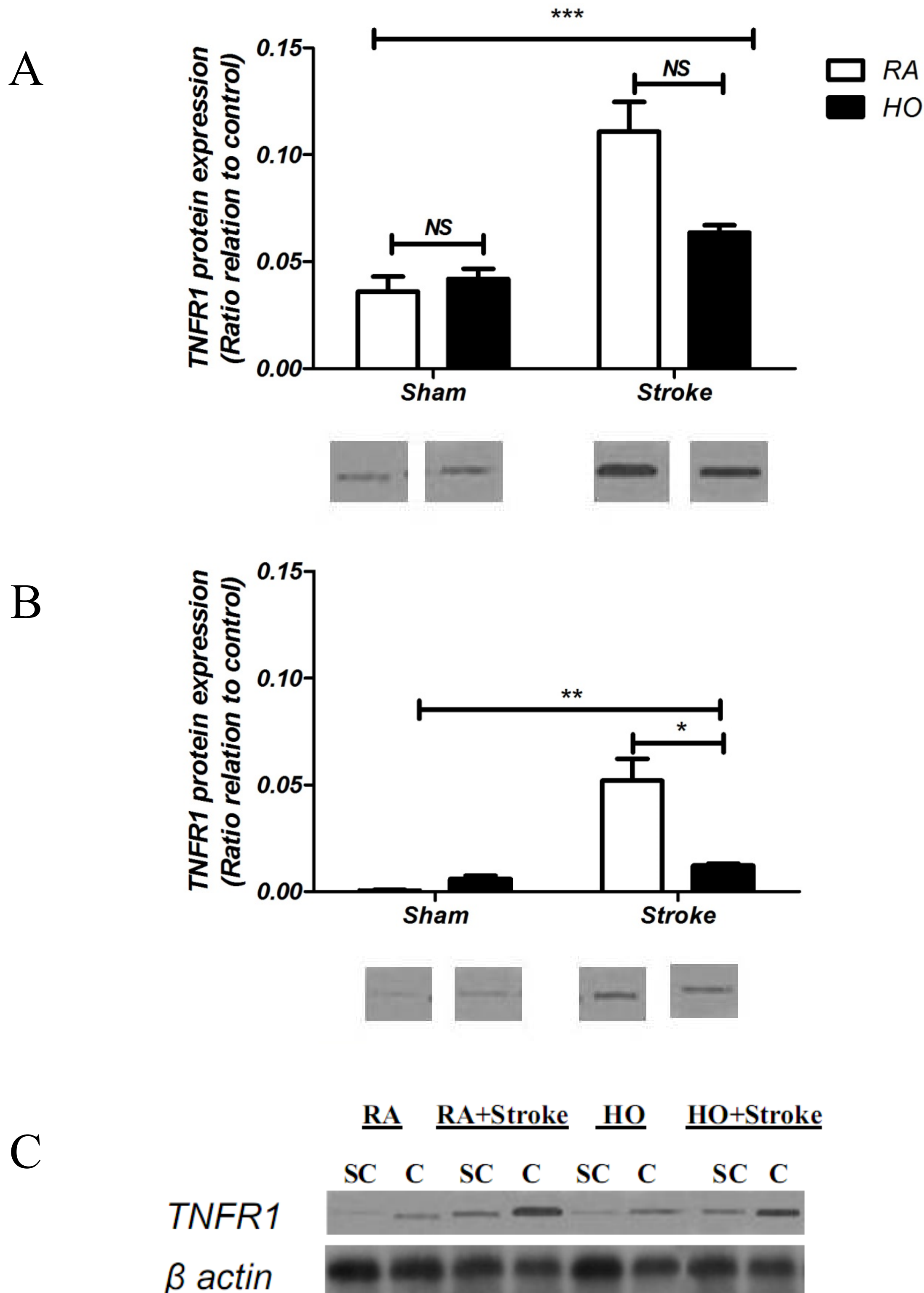

Fig.3. Hyperoxia effects on expression of TNFR1 in the cortex (A), subcortex (B) and desired bands (C). Hyperoxia (Ho); room air (RA); subcortex (SC); cortex (C). NS; non-significant. (n=6 for each group, data as mean ± SEM and *P<0.05, **P<0.01, ***P<0.001).

important role in decreasing NDS (Bigdeli et al., 2007). Hyperoxia also decreases toxicity via reduction of extracellular glutamate in the rat brain (Grabb et al., 2002). We have also previously demonstrated that preconditioning with prolonged and intermittent normobaric HO up-regulates glutamate transporters (EAAT1, EAAT2, EAAT3 and TACE) and serum TNFα levels in the rat brain (Bigdeli et al., 2009). The MCAO 'suture' model used here is a reliable and reproducible model (Longa et al.,

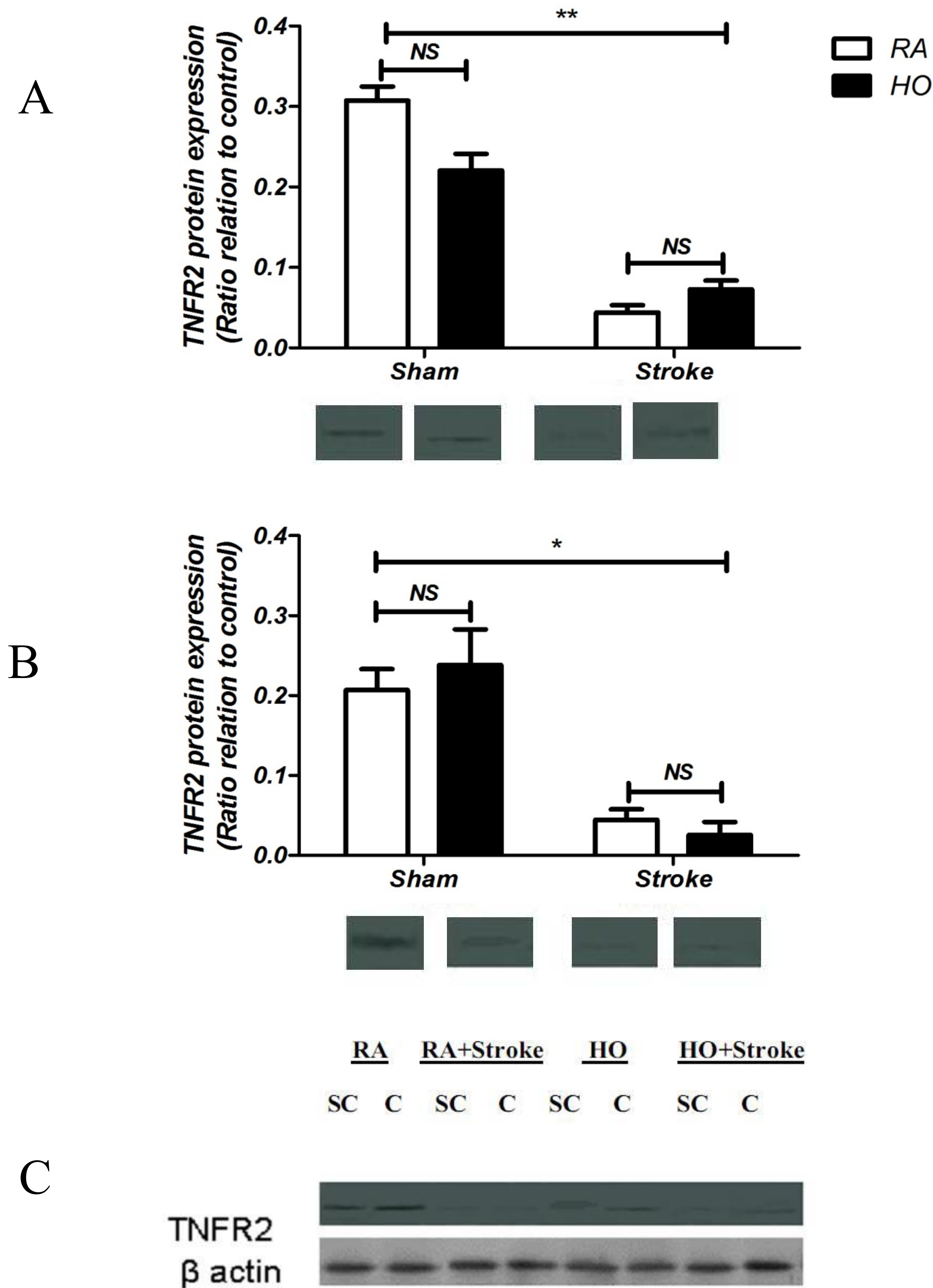

Fig.4. Hyperoxia effects on expression of TNFR1 in the cortex (A), subcortex (B) and desired bands (C). Hyperoxia (Ho); room air (RA); subcortex (SC); cortex (C). NS; non-significant. (n=6 for each group, data as mean ± SEM and $*P<0.05$, $**P<0.01$).

1989) which induces less subarachnoid haemorrhage and hypothalamically-induced hyperthermia than the intraluminal filament model (Dittmar et al., 2003).

According to research, normobaric HO ischemic tolerance can be done from two ways: a) via extracellular pathways and cytokines such as TNFα (Pradillo et al., 2006; Romera et al., 2004) and b) through the intracellular tracks and ROS increasing (Bigdeli and Mohagheghi, 2014; Weaver and Liu, 2015). Neuroprotection induced by HO shows specific adaptation responses that involve a number of cellular and biochemical changes, including

Table 1: ABG tests at the end of pre-treatment

Experimental groups	pH	PCO2 (mmHg)	PO2 (mmHg)	Blood pressure (mmHg)	Respiratory rate (Hz)
RA	7.3±0.01	39.8±1.7	94.9±8.6	87.32±75.09	1.38±0.01
In HO	7.2±0.01	40.9±1.4	358.5±41.9***	86±.42.2	1.58±0.89

RA: Room Air; InHO: Intermittent Hyperoxia

Table 2: The distribution of neurologic deficit score in each groups

No.	Experimental groups	NDS in each groups (N)					Total (N)	Statistical results (*P* value)
		0	1	2	3	4		
1	**RA**	0	4	12	5	1	24	
2	**In HO**	11	5	4	2	2	24	***P*<0.01**

NDS: neurologic deficit score; N: the number of cases in each groups; sig.: significant; RA: room air; InHO: intermittent hyperoxia

metabolic homeostasis and gene expression. Both exposures to HO and ischemic-reperfusion lead to the generation of excessive ROS. Oxidants generated during the brief period of highly elevated partial pressure of oxygen (PO2) are important for triggering many signalling systems that are more oxidant sensitive, including various kinases (such as p38MAPK) and transcription factors (such as HIF1α) (Alavian et al., 2012b; Bigdeli, 2011).

Our results showed that stroke groups significantly express higher level of TNFR1 in both cortex and subcortex areas. Our experiments in support of previous work showed that TNFα/TNFR1 has an important role in the CNS pathogenesis and primary demyelination by transgenic TNFα expression in glia cells (Probert et al., 1995). We also showed that normobaric hyperoxia in cortex had no significant effect on the expression of TNFR1, but HO caused a significant reduction of TNFR1 expression in subcortex. Yet no report of brain tolerance induction to ischemia through hyperoxia by TNFR1 has been published. Our results showed a significant interaction between hyperoxia and reduction of TNFR1 expression in cortex. This evidence suggests a protective role of hyperoxia-induced reduction in TNFR1 expression. The difference between the cortex and subcortex, probably are caused by the difference between brain cells or signalling pathways and expression of this receptor through hyperoxia may be varying at different brain areas that require further studies.

Results of this research showed that groups of stroke compared to sham groups had a significant decrease in the TNFR2 expression on both cortex and subcortex area. This finding is consistent with the previous works; so that in experiments on a group of neurons with TNFR2 gene deletion, large number of these cells were apoptotic (Yang et al., 2002). In another experiment with inhibition of TNFR2, neurons sensitivity to TNFα or hypoxia was increasing (Shen et al., 1997). So TNFR2 may play a critical role in the ability of neurons in response to injury.

Two TNFα receptors have conflicting activities so that TNFR2 on a way affiliated to phosphoinositide 3-kinase (PI3K), is involved on neuroprotection which is in contrast to the role of neuronal injury of TNFR1 (Marchetti et al., 2004). TNFR1 is also associated with cell death of neurons in the hippocampus in response to TNFα. While the inhibition of TNFR2, is driving of apoptosis (Yang et al., 2002). This confirms the strong antagonist activity of TNFRs in external stress or tissue injury. Neuroprotective effect of TNFR2 through the TNFR2-PI3K-Akt-NF-κB and durable activity of NF-κB is critical on stressors resistance (Chen and Goeddel, 2002; Marchetti et al., 2004). On Alzheimer, Parkinson, multiple sclerosis and stroke disease, TNFR2 has negative regulation and its expression decreases, which results is in decreased neuronal survival (Dong et al., 2015). But when a neuron becomes ill, TNFR1 active cell death

through the TNFα which is secreted by the surrounding glial cells. This is because the affinity of TNFα with TNFR1 is more than TNFR2 (Yang et al., 2002).

Studies have shown that after reperfusion, ROS increases and the increased ROS, increasing production of TNFα and other cytokines. TNFR2 caused activation of NF-κB on nerve cells. Then NF-κB increases expression of genes involved in ischemic preconditioning including glutamate transporters (Bigdeli et al., 2009). The result is adapting to difficult situations of ischemia and increase the survival of nerve cells. Bigdeli and Khoshbaten showed that increase in glutamate transporter type 3 in condition intermittent hyperoxia was parallel to the increase of serum TNFα concentration (Bigdeli and Khoshbaten, 2008).

In our work, hyperoxia compared with normoxia did not have significant effect on TNFR2 expression in cortex and subcortex; this suggests the neuroprotective effect of HO is independent of TNFR2 expression in the MCAO model.

Conclusion

In summary, this is the first report showing the TNFR1 and 2 expressions after normobaric HO in the rat brain and its neuroprotective role in ischemic tolerance. These data indicate the importance of TNFR1 expression in response to HO in subcortex and cerebral protection; whereas effect of HO on TNFR1 in cortex and its effect on TNFR2 in cortex and subcortex are independent in the MCAO model. Finally elucidation of other mechanisms that regulate the acquisition of brain ischemia tolerance may help in the development of effective methods of HO preconditioning to protect the brain or reduce ischemic injury.

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Conflict of interest

The authors have no conflict of interest to declare.

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