Applied & Translational Genomics 5 (2015) 30–32

Contents lists available at ScienceDirect

Applied & Translational Genomics

journal homepage: www.elsevier.com/locate/atg

Investigation of redox status in chronic cerebral hypoperfusion-induced neurodegeneration in rats

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ARTICLE INFO

Article history:
Received 23 April 2015
Accepted 31 May 2015

Keywords:
Chronic cerebral hypoperfusion
Antioxidant enzymes
2VO rats

ABSTRACT

Aging related reduction in cerebral blood flow (CBF) has been linked with neurodegenerative disorders including Alzheimer's disease and dementia. Experimentally, a condition of chronic cerebral hypoperfusion due to reduced CBF can be induced by permanent bilateral occlusion of common carotid arteries (2-vessel occlusion, 2VO) in rats. Since oxidative stress, leading to neuronal apoptosis and death, is one of the mechanisms, which is thought to play a significant role in chronic degenerative neurological disorders, the present study was planned to assess the ROS status by measuring the levels of anti-oxidant enzymes that might occur during chronic cerebral hypoperfusion. Antioxidant enzymes namely glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase were measured in the brain tissue at eight weeks of 2VO induction in rats. Results show significantly elevated levels of GPx, SOD, and catalase enzymes as compared with the control group. It is possible that compensatory rise in antioxidant enzymes occurs in response to increased oxidative stress following ischemic insult.

1. Introduction

The vascular hypothesis of Alzheimer's disease (AD) which was first proposed by de La Torre and Mussivand (1993), suggested that aging is the most fundamental risk factor for AD. This hypothesis is based on the observation that cerebral perfusion normally declines during aging and worsens in the presence of long standing vascular risk factors that further lower CBF until attaining a critical threshold level of cerebral hypoperfusion. In chronic cerebral hypoperfusion blood flow is insufficient to meet the metabolic demands of brain tissue (de la Torre, 2010). It has been well established that aging and Alzheimer's disease (AD) including dementia are associated with reduced cerebral blood flow (Farkas and Luiten, 2001). Data from Doppler and clinical studies show that both the regional cerebral perfusion rate and the flow velocity in the cerebral resistance vessels decrease in aging humans and AD patients as compared to age-matched healthy control (Claus et al., 1998; Krejza et al., 1999; Vriens et al., 1989). Similarly, Aanerud et al. (2012) reported results from PET studies in healthy volunteers, a decrease with age in CBF and cerebral metabolic rate of oxygen consumption ($CMRO_2$) and increase in oxygen extraction fraction (OEF) in areas known to be vulnerable in AD and PD.

For the establishment of chronic cerebral hypoperfusion (CCH) (as it occurs in human aging and AD) permanent bilateral common carotid artery occlusion (BCCAO) (2-vessel occlusion, 2VO) of rats has been introduced (Farkas et al., 2007). 2VO rats have been used successfully as a model of vascular dementia to study the role of cerebral hypoperfusion in neurodegenerative processes (Jing et al., 2015). However, the CBF (cerebral blood flow) pattern in 2VO rats is not identical to that occurring in aging or dementia in humans. Following induction of occlusion in 2VO rats, the CBF drops sharply and then begins to normalize after about 3 months due to compensatory and adaptive mechanisms. There is, however, a relatively long period of time (8 weeks to 12 weeks) during which the cerebral hypoperfusion in 2VO rats is comparable to that in aging humans (Choy et al., 2006). Cerebral hypoperfusion in 2 VO rats induced neuronal cell death in hippocampus, cerebral cortex, the white matter areas and the visual system (Farkas et al., 2004; Liu et al., 2006; Ohtaki et al., 2006), and learning and memory impairment (Cechetti et al., 2012; Xi et al., 2014), as well as acceleration of cerebral amyloid angiopathy and promotion of cortical microinfarct (Okamoto et al., 2012).

Chronic cerebral hypoperfusion-induced neurodegeneration is associated with the generation of reactive oxygen species (ROS), which is lethal to neurons at high concentration. The ROS in turn initiate lipid peroxidation, generating lipid peroxides that are degraded to reactive aldehyde products such as malondialdehyde (MDA) (Muralikrishna and Hatcher, 2006). Along with the increase in lipid peroxidation, the

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http://dx.doi.org/10.1016/j.atg.2015.05.004

activities of enzymatic antioxidants such as Cu/Zn-superoxide dismutase (Cu/Zn-SOD) or the concentrations of non-enzymatic antioxidants such as glutathione (GSH) decrease (Nita et al., 2001). However, increased lipid peroxidation and a decrease in antioxidants enzymes have primarily been associated with reperfusion after ischemia, which is very gradual in permanent 2VO model due to the flow compensation. There are suggestions that oxidative injury plays a role in Alzheimer's disease, Parkinson's disease, multiple sclerosis, schizophrenia, mitochondrial encephalopathies and brain injury due to ischemia and reperfusion (Mittal, 1999). However, not much is known about the possible role of oxidative stress in the pathogenesis of chronic cerebral hypoperfusion-induced neurodegeneration in 2VO rats.

The present study was, therefore, designed to evaluate the ROS status by measuring the levels of antioxidant enzymes like glutathione peroxidase (GDx), superoxide dismutase (SOD), and catalase in chronic cerebral hypoperfusion-induced neurodegeneration in 2VO rats.

2. Methods

The study was conducted on Wistar rats of either sex, weighing 200 to 250 g. The rats were obtained from the Universiti Sains Malaysia and were kept in standard plastic cages. During one week of acclimatization, rats were randomly distributed in pairs per cage. They were maintained on ad libitum food and water with 12/12 h light/dark cycle. All procedures were complied with the guidelines of the U.S. Public Health Service and NIH concerning the care and use of animals for experimentation as well as the guidelines and recommendations of Malaysian National Animal Welfare Foundation. The IIUM Ethical Committee approved all experimental procedures.

After one week of acclimatization, rats were randomly divided into two groups. Group A ($n = 7$) rats served as a control and they were not subjected to 2VO. Group B ($n = 7$) rats were subjected to permanent, bilateral common carotid arteries occlusion (2VO).

2.1. 2VO procedure in rats

Surgery was done as described before (Cechetti et al., 2010). Briefly, under full aseptic conditions, atropine sulfate (0.1 mg/kg) was given intramuscularly as a pre-anesthetic agent to prevent any respiratory distress. Rats were anesthetized with intraperitoneal injection of ketamine (90 mg/kg) and xylazine (10 mg/kg). A 2 cm ventral midline skin incision was made in the neck area just above the sternal bone. Following gentle tweezing of neck muscles, the carotid artery inside the carotid sheath was located on both sides and the common carotid arteries were carefully separated from the vagus nerve after carefully cutting the carotid sheath. The common carotid arteries were doubly ligated by sterilized silk suture just below the bifurcation into internal and external carotid arteries and the arteries were cut between the two ligatures. The incision was stitched and the wound treated with povidone iodine ('Betadine') solution. The body temperature was maintained throughout the surgical procedure and recovery period by heat lamp until full recovery from the general anesthesia. Intraperitoneal injection of buprenorphine (0.05 mg/kg) was administered to rats, which did not start water drinking on recovery from anesthesia due to pain in neck muscles tweezing during 2VO surgery.

At 8th week of 2VO surgery, Group A and Group B rats were sacrificed by euthanization with ether. The brain was quickly dissected out and rinsed with phosphate buffer saline (pH 7.4). The whole brain was crudely minced with a spatula and divided into three portions. Each portion was weighed and further processed. Three of the samples were homogenized respectively in 5–10 ml/g of 50 mM cold Tris–HCl buffer containing 5 mM EDTA and 1 mM DTT (pH 7.5) for GPx; 5–10 ml/g cold 20 mM HEPES buffer containing 1 mM EGTA, 210 mM mannitol and 70 mM sucrose (pH 7.2) for SOD; and 5–10 ml/g of cold 50 mM potassium phosphate buffer containing 1 mM EDTA (pH 7.0) for catalase estimation. Samples were centrifuged at 10,000× *g* for 15 min at 4 °C for GPx and catalase, 1500× *g* for 5 min at 4 °C for SOD. Supernatant was collected and stored at −80 °C.

2.2. Determination of antioxidant enzymes

GPx, SOD, and catalase enzymes were estimated with the help of Cayman Chemicals assay kits, USA. For GPx (catalogue no. 703102), SOD (catalogue no. 706002), and catalase (catalogue no. 707002) instructions from the respective catalogues were strictly adhered to.

2.3. Statistical analysis

Data were analyzed using Predictive Analytics Software (PASW version 18.0) and are presented here as mean ± SEM values. Comparison between the groups was done by independent *t*-test (Gaussian). Differences were designated as significant when $p < 0.05$ at 95% confidence interval.

3. Results

Antioxidant enzymes, GPx, SOD, and catalase activities were found to be significantly increased at 8 weeks after permanent bilateral carotid artery occlusion in rats. The mean value of GPx activity in the 2VO group was found to be 306.18 ± 2.85 nmol/min/ml, which was significantly higher ($p < 0.001$) when compared with the control group (241.67 ± 1.94 nmol/min/ml). Similarly, the mean SOD and catalase enzyme activities were significantly higher (0.159 ± 0.001 U/ml and 9.49 ± 0.25 nmol/min/ml, respectively; $p < 0.001$) as compared with the control group (0.113 ± 0.004 U/ml and 6.64 ± 0.431 nmol/min/ml, respectively; $p < 0.001$) as shown in Table 1.

4. Discussion

It is noteworthy that vascular dementia is the second most common cause of senile dementia (Kalaria et al., 2008). Oxidative stress has long been linked to the neuronal cell death that is associated with neurodegenerative disorders, including dementia. In AD brain, the levels of antioxidant enzymes were found to be decreased, with an associated increase in protein oxidation, lipid peroxidation, DNA oxidation, and ROS formation, strongly suggesting a role for oxidative stress in the pathogenesis of AD (Butterfield and Laderback, 2002; Markesbery, 1997; Sultana et al., 2006). Whether oxidative stress is a primary cause or merely a consequence of associated neuronal cell death is still an open question.

Permanent bilateral ligation of the common carotid arteries in rats is a chronic cerebral hypoperfusion model, which results in significant reduction of cerebral blood flow and neuronal damage. There is abundant evidence showing that free radicals are capable of mediating neuronal degeneration and death, and are possibly involved in the pathogenesis of neuronal death in neurodegenerative diseases (Muralikrishna and Hatcher, 2006; Sultana et al., 2006).

We already have reported a highly significant increase in the mean MDA concentration in 2VO rats as compared to the control group at 8th week (Saxena et al., 2011). In the present study, antioxidant enzymes namely GPx, SOD, and catalase activities were evaluated and compared with that of control group at 8th week after induction of 2VO in rats. Our results clearly show a significant increase in antioxidant enzymes namely GPx, SOD, and catalase in the brain. It is possible that in response to increased oxidative stress following ischemic insult, compensatory rise in antioxidant enzymes occurs. Similarly, Liao et al. (2004) reported that chronic cerebral hypoperfusion resulted in a significant increase in SOD, GPx activities, and MDA content compared to sham-operated group in rats (Liao et al., 2004). However, Mracskó et al. (2010) did not find any changes in the antioxidant enzyme MnSOD (Mracskó et al., 2010), while in our study a significant increase in SOD (and catalase) was seen at 8 weeks after 2VO. However, Mracskó

Table 1
Mean values of GPx, SOD, and catalase in the control and 2VO groups.

Test parameters	Control (n = 7)	2VO (n = 7)			
	Mean ± SEM	Mean ± SEM	df	t-stat	*P*-value
GPx activity (nmol/min/ml)	241.668 ± 1.941	306.179 ± 2.846	12	18.723	<0.001
SOD activity (U/ml)	0.113 ± 0.004	0.159 ± 0.001	12	11.213	<0.001
Catalase activity (nmol/min/ml)	6.637 ± 0.431	9.486 ± 0.255	12	5.68	<0.001

et al. reported regional as well as temporal differences in the changes in oxidative enzymes that follow hypoperfusion after 2VO wherein changes in one direction seen in the early phase may be reversed or normalized later on for a particular enzyme. Moreover, changes in oxidative/antioxidant mechanisms in one area (e.g. the cortex) do not correspond to changes in another area (hippocampus) implying thereby that different areas of the brain react in different ways and with varying time course. Since Mracsko et al. studied MnSOD levels only in the cortex and hippocampal regions while our data is from the whole brain, changes in other areas of the brain could possibly have contributed to this difference.

Increased lipid peroxidation and a decrease in antioxidants enzymes have primarily been associated with reperfusion after ischemia, which is very gradual in permanent 2VO model due to the flow compensation. However, in chronic cerebral hypoperfusion there might be an increase and/or decrease in antioxidant enzymes levels at different time intervals after induction of 2VO in rats. Few and conflicting reports are available regarding the status of antioxidant enzymes in 2VO rats. Therefore, more studies are needed to firmly establish the exact role of antioxidant enzymes in chronic cerebral hypoperfusion in rats. These preliminary data require confirmation, but it is currently considered that 2VO creates a permanent ischemic/oligemic condition serious enough to sustain continuous oxidative stress (probably in both the acute and chronic phases), which could very well be the reason for the persistent and progressive neuronal damage.

To date, there are no specific drugs to cure, delay or prevent vascular dementia. Hence, therapeutic strategies to modulate lipid peroxidation early throughout the course of the disease may be promising in slowing or possibly preventing neurodegenerative disorders.

Acknowledgments

This work was supported by grant from the Research Management Centre, International Islamic University Malaysia (EDW B 0905-281).

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