Neuroscience 135 (2005) 1217–1229

RESPONSE OF PURKINJE NEURONS TO HYPOBARIC HYPOXIC EXPOSURE AS SHOWN BY ALTERATION IN EXPRESSION OF GLUTAMATE RECEPTORS, NITRIC OXIDE SYNTHASES AND CALCIUM BINDING PROTEINS

C. KAUR,[a]* V. SIVAKUMAR,[a] G. SINGH,[b] J. SINGH[c] AND E. A. LING[a]

[a]*Department of Anatomy, Faculty of Medicine, Blk MD10, 4 Medical Drive, National University of Singapore, Singapore 117597*

[b]*Tan Tock Seng Hospital, 11 Jalan Tan Tock Seng, Singapore 308433*

[c]*Singapore Aeromedical Centre, 492 Airport Road, Singapore 539945*

Abstract—Hypobaric hypoxia is known to impair muscular coordination. It is not known whether hypobaric hypoxia causes any damage to the Purkinje neurons which may be responsible for impairment of muscular coordination. Expression of ionotropic glutamate receptors *N*-methyl-D-aspartate receptor subunit 1, amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid GluR2/3, calcium binding proteins and nitric oxide synthases in the Purkinje neurons was examined in rats exposed to hypobaric hypoxia. The mRNA expression of *N*-methyl-D-aspartate receptor subunit 1, GluR2, GluR3 and nitric oxide synthases [neuronal, endothelial and inducible] was upregulated at 3 h peaking at 24 h after the exposure. This was sustained up to 3 days; thereafter, it was comparable to the controls. Immunohistochemical analysis confirmed a marked expression of *N*-methyl-D-aspartate receptor subunit 1 and GluR2/3 at the above time intervals. Immunoexpression of calbindin-D28k (calbindin) and parvalbumin was intense in the soma of Purkinje neurons in the control rats. It was, however, drastically downregulated up to 3 days after exposure. At 3 days the neuronal dendrites showed intense expression of calbindin which returned to control levels at 7 days. Expression of neuronal nitric oxide synthase and inducible nitric oxide synthase was markedly upregulated from 3 h to 3 days whereas endothelial nitric oxide synthase expression, localized in the blood vessels and Purkinje neurons, remained elevated up to 24 h after the exposure. A progressive darkening of the Purkinje neuron cell bodies was observed at ultrastructural level up to 3 days but degenerating cells were not observed. A salient alteration was the dilation and stacking of smooth endoplasmic reticulum in the dendrites up to 14 days after the exposure. The present results suggest that hypobaric hypoxia leads to overexpression of *N*-methyl-D-aspartate receptor subunit 1 and GluR2/3 in Purkinje neurons that may be responsive to altered calcium levels as manifested by decreased expression of calcium binding proteins. This together with excess nitric oxide production may have led to transient ultrastructural changes. We propose that the functions of the Purkinje neurons may be altered in response to an acute exposure to hypobaric hypoxia resulting in impairment of motor coordination.

Key words: hypobaric hypoxia, cerebellum, Purkinje neurons, glutamate receptors, calcium binding proteins, nitric oxide synthases.

The cerebellum modulates motor control, posture and equilibrium. Maintenance of posture, equilibrium and locomotor coordination are impaired following damage to the cerebellum (Caston et al., 1998; Earhart and Bastian, 2001). Degenerative changes in the Purkinje neurons and failure of their normal activity are known be the underlying causes for various types of ataxias (Koeppen, 1991; Sasaki et al., 1998; Jeong et al., 2000). High altitude exposures are known to impair muscular coordination (McLennan and Ungersma, 1983) including tasks requiring eye–hand coordination (Ernsting and Sharp, 1986) and posture (Nordahl et al., 1998). Hypobaric hypoxia, which develops at high altitude as a consequence of reduced oxygen tension in the atmospheric air (Frisancho, 1975), is thought to be the underlying cause of impairment of muscular coordination and postural control (Ernsting and Sharp, 1986; Fraser et al., 1987). It is also known that muscular incoordination becomes greater with increasing altitude (Ernsting and Sharp, 1986). Ataxia associated with high altitude cerebral edema (HACE, Yarnell et al., 2000) and acute mountain sickness (AMS) has also been reported (Berghold, 2000). Although it has been postulated that postural ataxia might result from different hypoxia-related mechanisms (Baumgartner and Bartsch, 2002), the information on such mechanisms is scarce. As the Purkinje neurons in the cerebellum are known to be vulnerable to damage in hypoxic conditions (Woodruff-Pak et al., 1990), the present study was conducted to examine their response to an acute exposure of hypobaric hypoxia as at high altitude. Nitric oxide (NO), synthesized from the L-arginine by the family of nitric oxide synthase (NOS) enzymes (Garthwaite, 1991) has been implicated in the pathogenesis of brain injury from hypoxia–ischemia (Black et al., 1995). Since neuronal nitric oxide synthase (nNOS) is known to be expressed under various stressful conditions or injuries in the Purkinje neurons (Saxon and Beitz, 1996; Chen and Aston-Jones, 1994), the present study also sought to examine its expression in the Purkinje cells following the stress of hypobaric hypoxia.

*Corresponding author. Tel: +65-8743209; fax: +65-67787643.
E-mail address: antkaurc@nus.edu.sg (C. Kaur).

Abbreviations: AMPA, amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; NMDA, *N*-methyl-D-aspartate; NMDAR1, *N*-methyl-D-aspartate receptor subunit 1; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; SER, smooth endoplasmic reticulum.

0306-4522/05$30.00+0.00
doi:10.1016/j.neuroscience.2005.06.023

Activation of glutamate receptors particularly NMDA (*N*-methyl-D-aspartate) and AMPA (amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) following ischemic or hypoxic insults has been described as a possible triggering mechanism for neuronal death (Barenberg et al., 2001; Rothman and Olney, 1986; Choi, 1988: Nicholls and Attwell, 1990). Earlier studies have shown that glutamate acting via NMDA receptors activates nNOS (Garthwaite, 1991) and the production of NO by nNOS is closely related to the activation of NMDA receptors (Kiss and Vizi, 2001). In view of the above, we also aimed to examine whether there are any changes in the protein and mRNA expression of *N*-methyl-D-aspartate receptor subunit 1 (NMDAR1) and AMPA GluR2/3 receptors following hypobaric hypoxic exposures.

Brain hypoxia/ischemia triggers a cascade of events, possibly mediated by excitatory amino acids, yielding the activation of the Ca^{2+}-dependent NOS isoforms, i.e. nNOS and endothelial nitric oxide synthase (eNOS) increasing the production of NO (Bolanos and Almeida, 1999). In view of the above, besides nNOS, we also examined the expression of eNOS and inducible nitric oxide synthase (iNOS), in the cerebellum following the hypoxic exposure.

Purkinje neurons are rich in calcium and calcium buffering/sequestering systems including calbindin-D28k and parvalbumin (Katsetos et al., 2001). Calbindin-D28k is highly expressed in Purkinje neurons and acts as a cellular Ca^{2+} buffer. It has been hypothesized that perinatal hypoxia results in decreased concentration, or availability of calbindin-D28k in Purkinje neurons, thereby decreasing their buffering capacity and resulting in increase of intracellular Ca^{2+} (Katsetos et al., 2001) which may lead to neuronal degeneration. Parvalbumin is additionally found in a subpopulation of inhibitory interneurons, the stellate and basket cells (Schwaller et al., 2002) in the cerebellum. An increase in the intracellular Ca^{2+} has been found in the Purkinje neurons in seizures where the calbindin and parvalbumin concentrations were decreased (Kang et al., 2002) leading to degeneration of these cells. Keeping in view the important role of the calcium binding proteins we examined the expression of these proteins in the Purkinje neurons following the hypoxic exposure.

Along with the above, this study also sought to ascertain whether the Purkinje neurons undergo degeneration following hypobaric hypoxic insult. This takes into consideration results of an earlier study reporting dark cell degeneration of the Purkinje neurons in postnatal rats at ultrastructural level following hypoxic injury (Barenberg et al., 2001).

EXPERIMENTAL PROCEDURES

Fifty-four male Wistar albino rats weighing 200 g each were exposed to hypobaric hypoxia by placing them in a decompression (altitude) chamber (model 16M, Environmental Tectonics Corporation International, Southampton, PA, USA) at an atmospheric pressure of 350 mm Hg (equivalent to an altitude of 20,000 ft) for 2 h. The pO2 at this pressure is 73 mm Hg (pO_2 159 mm Hg at sea level). Another group of 10 rats of similar body weight was kept outside the chamber (pO_2 159 mm Hg) and was used as controls. In the handling and care of animals, the international guiding principles for research as stipulated by WHO Chronicle 39 (2): 51–56 (1985) and as adopted by the Laboratory Animals Centre, Animal Holding Unit, National University of Singapore were followed. Every effort was made to minimize the number of animals used and their suffering.

Immunohistochemistry

Rats exposed to hypobaric hypoxia were killed at 3, 24 h and 3, 7,14 and 21 days following exposure (n=4 at each time interval) along with the control rats (n=4). Following deep anesthesia with 7% chloral hydrate the rats were killed by perfusion with an aldehyde fixative composed of periodate lysine paraformaldehyde with a concentration of 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for nNOS, eNOS, iNOS, NMDAR1, GluR2/3, calbindin and parvalbumin immunohistochemistry. The cerebellum was removed and 40 μm thick coronal sections were cut and divided into seven sets. They were incubated with various antibodies as listed in Table 1, in phosphate buffered saline (PBS), respectively, for 16–20 h. Subsequent antibody detection was carried out by using Vectastain ABC kit (PK 4001 and PK 4002, Vector Laboratories, CA, USA) against mouse IgG with 3, 3′-diaminobenzidine tetrachloride (DAB) as a peroxidase substrate. For negative controls, some sections were incubated in a medium omitting the primary antibodies.

Real-time RT-PCR

Isolation of RNA. Wistar male rats (200–250 g, n=3/time point) were used in control as well as 3, 24 h, 3 and 7 days of hypoxic exposure. The animals were anesthetized with an i.p. injection of 7% chloral hydrate. The cerebellum was collected and stored at −80 °C until RNA extraction. Total RNA was isolated using RNeasy mini kit (Qiagen, CA, USA) and the quality and quantity of RNA were determined by Eppendorf Biophotometer (ratios of A260:A280 were >1.8).

Table 1. Antibodies used in the present study

Antibody	Host	Source	Dilution	Detection of
NMDAR1	Rabbit	Chemicon, CA, USA (AB-1516)	1:200	NMDA receptor subunit 1
GluR2/3	Rabbit	Chemicon, CA, USA (AB-1506)	1:200	AMPA glutamate receptors types 2 and 3
nNOS	Rabbit	BD Transduction Laboratories, CA, USA (610310)	1:500	nNOS
eNOS	Mouse	BD Transduction Laboratories, CA, USA (610296)	1:250	eNOS
iNOS	Mouse	BD Transduction Laboratories, CA, USA (610431)	1:1000	iNOS
Calbindin-D28k	Rabbit	SWant, Bellinzona, Switzerland (CB-38)	1:1000	Calcium binding proteins
Parvalbumin	Rabbit	SWant, Bellinzona, Switzerland (PV-28)	1:1000	Calcium binding proteins