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DISRUPTION OF CEREBELLAR CHOLINERGIC SYSTEM IN HYPOXIC NEONATAL RATS AND ITS REGULATION WITH GLUCOSE, OXYGEN AND EPINEPHRINE RESUSCITATIONS

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Abstract—Cholinergic system is important for respiratory control from the first days of life. Disturbances in cholinergic pathway due to early life stress like hypoxic shock can adversely affect the ventilatory response. The present study evaluates neonatal hypoxic insult mediated cholinergic disturbances and the role of glucose, oxygen and epinephrine resuscitation. The changes in total muscarinic, muscarinic M1, M2, M3 receptors and the enzymes involved in acetylcholine metabolism – cholineacetyl transferase and acetylcholine easterase in the cerebellum were analyzed. Hypoxic stress decreased cerebellar muscarinic receptor density with a decreased muscarinic M1, M2 and M3 receptor gene expression. The metabolic shift in the acetylcholine synthesis and release is indicated by the decreased cholineacetyl transferase mRNA expression and increased acetylcholine esterase gene expression. Glucose, acting as a precursor for acetyl choline synthesis and an immediate energy source, helps in reversing the cholinergic disturbances in hypoxic neonates. The limitation of immediate oxygenation and epinephrine administration in ameliorating cholinergic disturbances in hypoxic neonates was also reported. This will help in devising a better resuscitation program for the management of neonatal hypoxia.

Key words: neonatal hypoxia, cholineacetyl transferase, acetylcholine easterase, muscarinic receptor, glucose, oxygen.

INTRODUCTION

The perinatal period is a critical time which determines the fundamental changes in the cardiorespiratory status of the baby. Respiratory gas exchange, formerly a placental function, must be established by the lungs within minutes after birth. Therefore, frequent and serious difficulties in cardiorespiratory adaptation in the perinatal and neonatal periods are not surprising. Respiration is a highly integrated process that involves a complex network of interplay between the brain, spinal cord, cranial and spinal nerves, diaphragm, intercostal muscles, laryngeal and pharyngeal structures, lungs and the vasculature. It also involves diverse sets of neurotransmitters, neuromodulators, receptors, second messengers and transcription factors. Hypoxia occurring before or shortly after birth is a major cause of life-threatening injury and lifelong disability (du Plessis and Volpe, 2002; Schubert et al., 2005). Hypoxia results in multi-organ failure and structural/functional damage especially devastating to the cardiovascular, renal, gastrointestinal and central nervous systems (Shah et al., 2004; Vento et al., 2005). Hypoxic brain injury is very complex and has different neuropathological manifestations depending on the maturity of the newborn. Thus, understanding the diagnosis, pathogenesis, resuscitation and treatment of those infants suffering hypoxic brain injury is paramount to reduce disability, improving survival and enhancing quality of life.

Muscarinic cholinergic mechanisms are important for respiratory control from the first days of life. Respiratory stimulation might be the predominant, although not the exclusive, effect of muscarinic receptor activation in the respiratory neuronal network. Consequently, any alteration in baseline breathing resulting in a respiratory depression can be attributed to muscarinic receptors and originating within this network. The cerebellum significantly differs with respect to ischemia and hypoxia, this response being directly related to the duration and intensity of the injury. The cerebellum could cover the eventual need for nitric oxide during the hypoxia, boosting the nitric oxide synthase activity, but overall ischemia would require de novo protein synthesis, activating the inducible nitric oxide synthase to cope with the new situation (Rodrigo et al., 2004). Further, acetyl choline is released from the cerebellum during hypoxia (Fitzgerald et al., 2000). Hypoxic insult resulted in considerable neurocytological deficits of the Purkinje cells and altered glial fibrillary acid protein immunoreactivity in the fetal cerebellum. Acetylcholine (ACh) modulates neurons involved in the generation of respiratory frequency and pattern (Shao and Feldman, 2005) and influences chemoreceptor responses (Nattie, 1999). Cholinergic stimulation of the pontine reticular formation alters respiratory regulation (Kubin and Fenik, 2004), slows respiratory rate (Lydic and Baghdoyan, 2003) and suppresses ventilatory responses via connections of this cholinoceptive region to respiratory nuclei (Lee et al.,

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Abbreviations: ACh, acetylcholine; AChE, acetylcholine esterase; ANOVA, analysis of variance; ChAT, choline acetyltransferase; CoA, choline *O* acetyltransferase; QNB, quinuclidinylbenzilate; ROS, reactive oxygen species; S.E.M., standard error of the mean.

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1995). Immunocytochemical study of the cerebellar cortex showed that Purkinje cells containing acetylcholine and its metabolic enzymes are most common in the flocculondular lobe and they are randomly distributed without a clear pattern. Cholinergic modulation of the neuronal excitability in the pre-Bötzinger complex (preBötC) affects respiratory rhythm and is mediated by both muscarinic acetylcholine receptors (AChRs) and nicotinic AChRs (nAChRs) (Shao and Feldman, 2001; Bellingham and Ireland, 2002). Thus the study of cholinergic muscarinic receptor alterations in the cerebellum can throw light on the significance of neurotransmission in hypoxic ventilatory response.

Choline acetyltransferase (ChAT, acetyl CoA:choline *O* acetyltransferase, EC 2.3.1.6), the enzyme responsible for the biosynthesis of acetylcholine, is the most specific indicator for monitoring the functional state of cholinergic neurons in the central and peripheral nervous systems. ChAT immunoreactivity was reported in cerebellar mossy fibers and around Purkinje cells. The reduction of ChAT is correlated with the severity of dementia and pathologic changes (Rodrigo et al., 2005). The termination of cholinergic nerve impulse transmission is accomplished through the degradation of acetylcholine into choline and acetyl CoA by acetylcholine esterase (AChE, EC 3.1.1.7) (Weihua et al., 2000). Therefore, expression of ChAT and AChE will provide us with an indication for understanding the physiologic states of cholinergic neurons. Mild hypoxia, which impairs memory and judgment, decreases ACh synthesis. The decreases in glucose incorporation into ACh and into the amino acids with hypoxic hypoxia (15% or 10% O_2) or hypoxic hypoxia with 5% CO_2 were very similar to those with the two lowest levels of anemic hypoxia. Thus, any explanation of the brains' sensitivity to a decrease in oxygen availability must include the alterations in the metabolism of the amino acid neurotransmitters as well as Ach (Gibson and Peterson, 1981).

The present study tries to understand the cerebellar cholinergic disturbances in neonatal hypoxic condition and evaluated the effect of 100% oxygen, glucose and epinephrine resuscitation methods in encountering this cholinergic disturbance. Histological analysis using TOPRO-3 staining was done to understand the severity of hypoxic shock and alterations in the cholinergic function was studied by muscarinic receptor assays and the expression of ChAT and AChE. The understanding of cholinergic regulation of neonatal hypoxia will help in devising better treatment methods.

EXPERIMENTAL PROCEDURES

Animals

Neonatal Wistar rats were purchased from Amrita Institute of Medical Sciences, Cochin. All groups of neonatal rats were maintained with their mothers under optimal conditions – 12-h light and 12-h dark periods and were fed standard food and water *ad libitum*. All animal care and procedures were taken in accordance with the institutional, National Institute of Health guidelines and Committee for the Purpose of Control and Supervision of Experiments on Animals, India. (CPCSEA) guidelines.

Induction of acute hypoxia in neonatal rats and tissue preparation

Wistar neonatal rats 4-days old were used for the experiments and were grouped into seven as follows: (i) C – control neonatal rats exposed to atmospheric air (20.9% oxygen) for 30 min (ii) Hx – hypoxia neonatal rats exposed to 2.6% oxygen for 30 min (Hx); (iii) Hx + G – neonatal rats treated immediately after hypoxic insult with 560 mM glucose (10% glucose; 500 mg/kg body wt.) i.p. (iv) Hx + O – neonatal rats treated immediately after hypoxic insult with 100% oxygen for 30 min (v) Hx + G + O – neonatal rats treated immediately after hypoxic insult with 560 mM glucose i.p. followed by 100% oxygen for 30 min (vi) Hx + G + E + O – neonatal rats treated immediately after hypoxic insult with 560 mM glucose i.p. and 0.46 μM epinephrine (0.10 μg/kg body wt. i.p.) followed by 100% oxygen for 30 min (vii) Hx + E – neonatal rats treated immediately after hypoxic insult with 0.46 μM epinephrine (0.10 μg/kg body wt.) i.p. Respiratory rate was measured as breaths/minute in all the experimental groups. Skin color and behaviur of the neonatal rats were noticed to confirm the physical changes under hypoxic stress.

Control and experimental neonatal rats were sacrificed by decapitation on postnatal day 14. The cerebellum was dissected out quickly over ice according to the procedure of Glowinski and Iversen, 1966 and was stored at −80 °C for various experiments.

Histological analysis of hypoxic cerebellum using TOPRO-3 staining

The anaesthetized hypoxic and control neonatal rats were transcardially perfused with Phosphate buffer saline (PBS) (pH – 7.4) followed by 4% paraformaldehyde in PBS. After perfusion the brain was dissected out and fixed in 4% paraformaldehyde for 1 h and then equilibrated with 30% sucrose solution in PBS (0.1 M). Ten micrometer sagittal sections of the cerebellum were taken using Cryostat (Leica, CM1510 S). TOPRO-3 stain (diluted 1:1000 in PBS) was added and kept for 10 min at room temperature. The sections were observed and photographed using confocal imaging system (Leica TCS SP 5).

Total muscarinic receptor binding assay

The total muscarinic receptor binding assay in the cerebellum was done according to the modified procedure of Yamamura and Synder (1981). Total muscarinic binding assay was done using 0.1–2.5 nM of [^{3}H] quinuclidinylbenzilate (QNB) in a total incubation volume of 200 μl with 200–250 μg protein concentration. The non-specific binding was determined using 100 μM atropine. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. Protein was measured by the method of Lowry et al., 1951.

Linear regression analysis of receptor binding data for Scatchard plots

The data were analyzed according to Scatchard (1949). The binding parameters, maximal binding (B_{max}) and equilibrium dissociation constant (K_d), were derived by linear regression analysis by plotting the specific binding of the radioligand on *X*-axis and bound/free on *Y*-axis. The maximal binding is a measure of the total number of receptors present in the tissue