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Decreased cholinergic function in the cerebral cortex of hypoxic neonatal rats: Role of glucose, oxygen and epinephrine resuscitation

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ABSTRACT

Molecular processes regulating cholinergic functions play an important role in the control of respiration under hypoxia. Cholinergic alterations and its further complications in learning and memory due to hypoxic insult in neonatal rats and the effect of glucose, oxygen and epinephrine resuscitation was evaluated in the present study. Receptor binding and gene expression studies were done in the cerebral cortex to analyze the changes in total muscarinic receptors, muscarinic M1, M2, M3 receptors and the enzymes involved in acetylcholine metabolism – choline acetyltransferase and acetylcholine esterase. Neonatal hypoxia decreased total muscarinic receptors with reduced muscarinic M1, M2 and M3 receptor genes. The reduction in acetylcholine metabolism is indicated by the down regulated choline acetyltransferase and up regulated acetylcholine esterase expression. These cholinergic disturbances were reversed to near control in glucose resuscitated hypoxic neonates. The adverse effects of immediate oxygenation and epinephrine administration are also reported. This has immense clinical significance in establishing a proper resuscitation for the management of neonatal hypoxia.

1. Introduction

Hypoxic brain injury is very complex and has different neuropathological manifestations depending on the maturity of the newborn (Schubert et al., 2005). Brain is of special interest for hypoxia studies as it is extremely sensitive to reductions in oxygen supply. The brain damage occurs within a few minutes of hypoxia and result in severe and complex disabilities or death (Slavin et al., 1994). Mild hypoxia, which impairs memory and judgment, decreases acetylcholine (ACh) synthesis, but not the levels of ATP or the adenylate energy charge. 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 anaemic hypoxia (Gibson and Peterson, 1981). 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. The synthesis of ACh takes place in the axonal terminals and is catalyzed by the cytosolic enzyme choline acetyltransferase (ChAT) (van der Zee and Luiten, 1999). The termination of nerve impulse transmission is accomplished through the degradation of acetylcholine into choline and acetyl CoA by acetylcholine esterase (AChE) (Weihua et al., 2000). The expression of ChAT and AChE can be taken as an index of cholinergic pathway.

Acetylcholine acting through muscarinic receptors is involved in the central control of respiration (Haji et al., 2000). Pharmacological evidence suggests that the muscarinic M1 and M3 receptor subtypes play a predominant role in respiratory control, primarily based on studies in the neonatal rodent brain stem preparation *in vitro* (Burton et al., 1994) and in the adult cat (Nattie and Li, 1990). The majority of respiratory neurons are cholinoceptive and are either excited or depressed by ACh or ACh agonists applied by iontophoresis *in vivo*, the proportion of depressed neurons being higher in anesthetized animals than in decerebrated preparations (Morin-Surun et al., 1984). It was shown that ACh increase respiratory frequency through muscarinic M3 receptor activation of rhythm-generating neurons in the pre-Bötzinger complex in the medullary slice *in vitro* (Shao and Feldman, 2000). Signaling through muscarinic M1 and M3 AChRs promote accumulation and transcriptional activation of HIF-1α. Muscarinic acetylcholine signals activate HIF-1 by both stabilization and synthesis of HIF-1α and by inducing the transcriptional activity of HIF-1α (Hirota et al., 2004). Serious hypoxic–ischemic events are known to have an adverse impact on cognitive function (Menkes et al., 1995). Exposure to hypoxia causes alterations of acetylcholine metabolism and receptor level which in turn results cognitive impairment in terms of learning and memory.

The response of central nervous system to oxygen deprivation/hypoxia are vital in revealing mechanisms that participate in coordinated behaviour of respiratory and vasomotor activities

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(Acker and Acker, 2004). Disruption of the cholinergic innervations during postnatal development results in delayed cortical neuronal development and permanent changes in cortical cytoarchitecture and cognitive function (Angela and Russell, 2004). Approaches to prevent/treat cerebral hypoxic damage in neonates (Tuor et al., 1996) are important for neonatal intensive care unit. The present study focused on the cortical cholinergic disturbances which can contribute to developmental and cognitive deficits.

In the present study, we evaluated the cholinergic disturbances in the cerebral cortex of hypoxic neonatal rats by studying the alterations in the muscarinic receptor function and the gene expression of muscarinic receptor M1, M2, M3 and enzymes involved in acetylcholine metabolism. The learning and memory deficit caused by neonatal hypoxic insult in the later stages of life was also examined. The effectiveness of various resuscitation methods like administration of 100% oxygen and intravenous fluids like 10% glucose and 0.10 g/kg body wt epinephrine alone and in combinations in reversing the cholinergic alterations in neonatal hypoxia was analyzed to understand the neuroprotective role of glucose supplementation. Understanding the detailed mechanisms involved in hypoxic responses may provide general insight into a better resuscitation program to overcome neonatal hypoxia thereby ensuring a better intellect in the later stages of life.

2. Materials and methods

Bio chemicals used in the present study were purchased from Sigma Chemical Co., St. Louis, USA. All other reagents of analytical grade were purchased locally. Quinuclidinyl benzilate, L-[benzilic-4,4′-3H], ([^{3}H]QNB), Sp. Activity 42 Ci/mmol was purchased from NEN Life Sciences Products Inc., Boston, U.S.A. Tri-reagent kit was purchased from MRC, USA. Real Time PCR Taqman probe assays on demand were from Applied Biosystems, Foster City, CA, USA.

Neonatal Wistar rats were purchased from Amrita Institute of Medical Sciences, Cochin. Neonatal rats of 3 days old were weighed and used for experiments. Induction of hypoxia and supplementation of glucose, oxygen and epinephrine were done according to the procedure of Paulose et al. (2007). All groups of neonatal rat 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 CPCSEA guidelines.

2.1. Induction of acute hypoxia in neonatal rats and tissue preparation

Wistar neonatal rats of 4-days old (body weight, 6.06 ± 0.45 g) were used for the experiments and were grouped into seven as follows: (i) Control neonatal rats were given atmospheric air (20.9% oxygen) for 30 min (C); (ii) Hypoxia was induced by placing the neonatal rats in a hypoxic chamber provided with 2.6% oxygen for 30 min (Hx); (iii) Hypoxic neonatal rats were injected 10% dextrose (500 mg/kg body wt) intra-peritoneally (i.p.) (Hx + G). (iv) Hypoxic neonatal rats were supplied with 100% oxygen for 30 min (Hx + O); (v) Hypoxic neonatal rats were injected 10% dextrose (500 mg/kg body wt. i.p.) and treated with 100% oxygen for 30 min (Hx + G + O). (vi) Hypoxic neonatal rats were injected 10% dextrose (500 mg/kg body wt), epinephrine (0.1 μg/kg body wt. i.p.) and treated with 100% oxygen for 30 min (Hx + G + E + O) (vii) Hypoxic neonatal rats were injected with epinephrine (0.10 g/kg body wt i.p.) (Hx + E). Body weights were measured before the experiment.

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

2.2. Total muscarinic receptor binding study in the cerebral cortex

The total muscarinic receptor binding assay in the cerebral cortex was done according to the modified procedure of Yamamura and Synder (1981). Total muscarinic binding parameter assays were done using 0.1–2.5 nM of [^{3}H]quinuclidinylbenzilate (QNB) in a total incubation volume of 250 μl with 200–250 μg protein concentration. The non-specific binding was determined using 100 μM muscarinic general antagonist, 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).

2.3. Receptor data analysis

The data was 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 and the equilibrium dissociation constant is the measure of the affinity of the receptors for the radioligand. The K_d is inversely related to receptor affinity.

2.4. Analysis of gene expression using Real Time PCR

RNA was isolated from the cerebral cortex of experimental neonatal rats using the Tri-reagent (MRC, USA). Total cDNA synthesis was performed using ABI PRISM cDNA archive kit in 0.2 ml microfuge tubes. The reaction mixture of 20 μl contained 0.2 μg total RNA, 10× RT buffer, 25× dNTP mixture, 10× random primers, MultiScribe RT (50 U/μl) and RNase free water. The cDNA synthesis reactions were carried out at 25 °C for 10 min and 37 °C for 2 h using an Eppendorf Personal Cycler. Real-time PCR assays were performed in 96-well plates in ABI 7300 real-time PCR instrument (Applied Biosystems). The primers and probes were purchased from Applied Biosystems, Foster City, CA, USA. The TaqMan reaction mixture of 20 μl contained 25 ng of total RNA derived cDNAs, 200 nM each of the forward primer, reverse primer and TaqMan probe for muscarinic M1 receptor (Rn 00589936_s1), muscarinic M2 receptor (Rn 02532311_s1), muscarinic M3 receptor (Rn 00560986_s1), choline acetyltransferase (Rn 01453446_m1), acetylcholine esterase (Rn 00596883_ m1) and endogenous control β-actin and 12.5 μl of Taqman 2X Universal PCR Master Mix (Applied Biosystems) and the volume was made up with RNase free water. The following thermal cycling profile was used (40 cycles): 50 °C for 2 min, 95 °C for 10 min, 95 °C for 15 s and 60 °C for 1 min.

Fluorescence signals measured during amplification were considered positive if the fluorescence intensity was 20-fold greater than the standard deviation of the baseline fluorescence. The ΔΔCT method of relative quantification was used to determine the fold change in expression. This was done by normalizing the resulting threshold cycle (CT) values of the target mRNAs to the CT values of the internal control β-actin in the same samples ($\Delta CT = CT_{Target} - CT_{\beta\text{-actin}}$). It was further normalized with the control ($\Delta\Delta CT = \Delta CT - CT_{Control}$). The fold change in expression was then obtained as ($2^{-\Delta\Delta CT}$) and the graph was plotted using $\log 2^{-\Delta\Delta CT}$.

2.5. Spatial recognition memory assessment by Y maze

Y-maze is a simple 2-trial recognition test for measuring spatial recognition memory. It is based on the innate tendency of rodents