Regulation of Second Messenger Signaling in Hypoxic Neonatal Rats: Effect of Glucose, Oxygen and Epinephrine Resuscitation

Thoppil R Anju [1], and Cheramaduthikudiyil S Paulose [2]

***Abstract*—** Cell signaling via second messengers plays an important role in control of respiration under hypoxia. Second messenger alterations and its further complications in respiration due to neonatal hypoxic insult and the effect of glucose, oxygen and epinephrine resuscitation were evaluated in the present study. Second messenger assays and gene expression studies on CREB and phospholipase C were done in corpus striatum to analyze changes in second messenger signaling cascade. Neonatal hypoxia increased the concentration of IP3, cAMP and cGMP in the corpus striatum. The gene expression of downstream transcription factors in cell signaling - CREB was down regulated and phospholipase C was up regulated in neonatal hypoxic rats. These 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 management of neonatal hypoxia.

***Keywords*—** cAMP, cGMP, CREB, IP3, phospholipase C.

I. Introduction

HYPOXIA in newborns is a major cause of pediatric mortality and morbidity and causes brain damage resulting in life-long neurobehavioral handicaps. Hypoxia activates the expression of a number of genes and alters the neurotransmission and receptor expressions. Neonatal brain is highly sensitive to reduction in oxygen supply. Second messengers relay signals received at receptors on the cell surface to target molecules in the cytosol and/or nucleus. Three major classes of second messengers are cyclic nucleotides (e.g., cAMP and cGMP), inositol trisphosphate (IP_3) and diacylglycerol (DAG), calcium ions (Ca^{2+}). The IP3 induced Ca^{2+} signaling plays a crucial role in the control of diverse physiological processes such as contraction, secretion, gene expression and synaptic plasticity [1]. Hypoxia results in a modification of the binding characteristics of the neuronal nuclear membrane inositol tetrabisphosphate (IP4) and inositol triphosphate (IP3) receptors. Mishra and Delivoria-Papadopoulos (2004) observed an IP4- as well as IP3-dependent increase in nuclear Ca^{2+} influx with increasing cerebral tissue hypoxia, suggesting a hypoxia-induced modification of the nuclear membrane IP4 and IP3 receptors [2].

cGMP generation has been associated with neurotransmission [3]. cGMP effects are primarily mediated by the activation of cGMP-dependent protein kinases (PKGs). The most extensively studied cGMP signal transduction pathway is that triggered by nitric oxide (NO) [4]. NO–cGMP signaling is mechanistically involved in a number of animal models for learning and behavior, e.g. object recognition and passive avoidance [5].

Cyclic nucleotide pathways cross talk to modulate each other's synthesis, degradation and actions. Increased cGMP increase the activity of cGMP stimulated PDE2 to enhance hydrolysis of cAMP, or it inhibit the PDE3 family and decrease the hydrolysis of cAMP [6]. The cAMP responsive element binding protein (CREB) is a nuclear protein that modulates the transcription of genes with cAMP responsive elements in their promoters. Increases in the concentration of either Ca^{2+} or cAMP trigger the phosphorylation and activation of CREB. Genetic and pharmacological studies in mice and rats demonstrate that CREB is required for a variety of complex forms of memory, including spatial and social learning, thus indicating that CREB is a universal modulator of processes required for memory formation [7]. Beitner-Johnson and Millhorn (1998) reported that physiological reduction in O_2 levels induces a functional phosphorylation of CREB at Ser133 via a novel signaling pathway. Thus a proper understanding of the regulation of second messenger pathways under hypoxic stress will help in devising better resuscitation methods to combact hypoxia [8].

The present study was designed to investigate the alterations in second messenger signalling due to neonatal hypoxia and its regulation by various resuscitation methods like administration of 100% oxygen and intravenous fluids like 10% glucose and 0.10μg/Kg body wt epinephrine alone and in combinations. IP3, cAMP and cGMP content in the corpus striatum of hypoxic neonatal rats and various resuscitation methods were analysed along with the gene expression studies using Real Time PCR for CREB expression. The study tries to pin point a better resuscitation programme for neonatal hypoxic care by studying the impact of various resuscitation methods in combating the second

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messenger alterations evoked by hypoxia.

II. Materials And Methods

Tri-reagent kit was purchased from MRC, USA. Real Time PCR Taqman probe assays on demand were from Applied Biosystems, Foster City, CA, USA. Bio chemicals used in the present study were purchased from Sigma Chemical Co., St. Louis, USA.

All groups of neonatal rat were maintained with their mothers under optimal conditions – 12 hour light and 12 hour 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.

Induction of Acute Hypoxia in Neonatal Rats and Tissue preparation

Wistar neonatal rats of 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 minutes (ii) Hx - Hypoxia neonatal rats exposed to 2.6% oxygen for 30 minutes (Hx); (iii) Hx+G- Neonatal rats treated immediately after hypoxic insult with 560mM glucose (10% glucose; 500mg/ Kg body wt) intra-peritoneally (i.p.). (iv) Hx+O - Neonatal rats treated immediately after hypoxic insult with 100% oxygen for 30 minutes (v) Hx+G+O - Neonatal rats treated immediately after hypoxic insult with 560mM glucose i.p. followed by 100% oxygen for 30 minutes (vi) Hx+G+E+O - Neonatal rats treated immediately after hypoxic insult with 560mM glucose i.p. and 0.46μM epinephrine (0.10μg/Kg body wt. i.p.) followed by 100% oxygen for 30 minutes (vii) Hx + E - Neonatal rats treated immediately after hypoxic insult with 0.46μM epinephrine (0.10μ g/Kg body wt) i.p.

Control and experimental neonatal rats were sacrificed by decapitation on postnatal day 14. The corpus striatum was dissected out quickly over ice according to the procedure of Glowinski and Iversen (1966) [9] and was stored at -80°C for various experiments.

IP3, cGMP and cAMP content of control and experimental rats in vivo

Corpus striatum was homogenised in a polytron homogeniser in 50mM Tris-HCl buffer, pH.7.4, containing 1mM EDTA to obtain a 15% homogenate. The homogenate was then centrifuged and the supernatant was used for IP3 assay using [^{3}H]IP3 Biotrak Assay System kit, cGMP assay using [^{3}H]cGMP Biotrak Assay System kit and cAMP assay using [^{3}H]cAMP Biotrak Assay System kit. IP3, cGMP and cAMP concentration (picomoles/tube) in the samples was determined by interpolation from respective standard curves.

Analysis of gene expression of CREB and phospholipase C using Real Time PCR.

RNA was isolated from the corpus striatum using Tri-reagent. 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 CREB (Rn 00578826_m1), Phospholipase C (Rn 01647142) 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 $2^{-\Delta\Delta CT}$.

Statistical analysis

The equality of all the groups was tested by the analysis of variance (ANOVA) technique for different values of p. Further the pair wise comparisons of all the experimental groups were studied using Students-Newman-Keuls test at different significance levels. The testing was performed using GraphPad Instat (Ver. 2.04a, San Diego, USA) computer program.

III. Results

IP3 content in the corpus striatum of experimental groups of neonatal rats

The IP3 content in the corpus striatum increased significantly ($p<0.001$) in Hx, Hx+O, Hx + E and Hx+G+E+O compared to control. Glucose treatment to hypoxic rats – Hx+G, Hx+G+O significantly reversed ($p<0.001$) the IP3 levels to near control (Fig. 1).

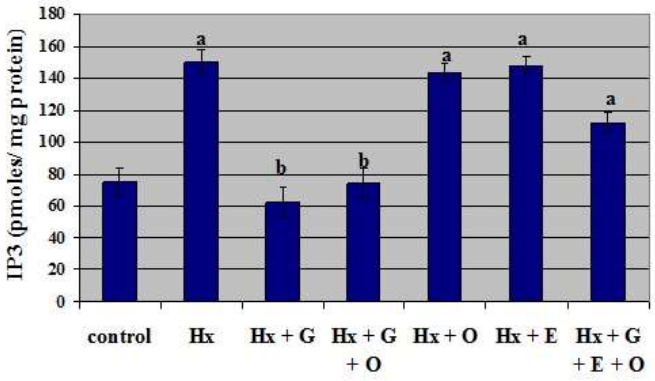

Fig. 1 IP3 content in the corpus striatum was analyzed using [^{3}H] IP3 Biotrak Assay System kit. Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4 - 6 neonatal rats. [a] p<0.001 when compared to Control. [b] p<0.001 when compared to Hx.

cGMP content in the corpus striatum of experimental groups of neonatal rats

The cGMP content in the corpus striatum increased significantly (p<0.001) in Hx, Hx+O, Hx + E and Hx+G+E+O compared to control. Glucose treatment to hypoxic rats – Hx+G (p<0.001), Hx+G+O (p<0.001) significantly reversed the cGMP content to near control (Fig. 2).

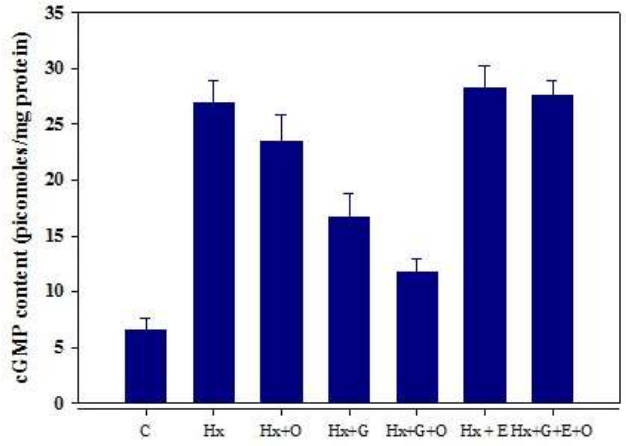

Fig. 2 Cyclic GMP content in the corpus striatum was analyzed using [3H]cGMP Biotrak Assay System kit. Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4 - 6 neonatal rats. a p<0.001 when compared to Control. b p<0.001 when compared to Hx.

cAMP content in the corpus striatum of experimental groups of neonatal rats

The cAMP content in the corpus striatum increased significantly (p<0.001) in Hx, Hx+O, Hx + E and Hx+G+E+O compared to control. Glucose treatment to hypoxic rats – Hx+G, Hx+G+O significantly reversed (p<0.001) the cAMP levels to near control (Fig. 3).

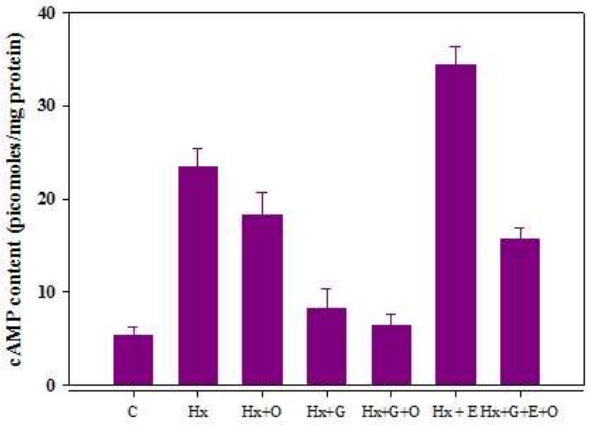

Fig. 3 Cyclic AMP content in the corpus striatum was analyzed using [3H]cAMP Biotrak Assay System kit. Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4 - 6 neonatal rats. a p<0.001 when compared to Control. b p<0.001 when compared to Hx.

Gene expression study of CREB mRNA using Real Time PCR analysis.

The gene expression of transcription factor CREB mRNA showed a significant down regulation (p<0.001) in the hypoxic group compared to control. In Hx + G, Hx + G + O and Hx + O there was a significant reversal (p<0.001) of the CREB expression to near control. Epinephrine resuscitated groups, Hx + E and Hx + G + E + O, showed no significant reversal in the gene expression to near control. (Table. 1).

TABLE I
REAL TIME PCR AMPLIFICATION OF CREB MRNA FROM THE CORPUS STRIATUM OF CONTROL AND EXPERIMENTAL NEONATAL RATS

Experimental groups	Log RQ
Control	0
Hx	-2.57 ± 0.10 [a]
Hx + G	0.33 ± 0.09 [b]
Hx + G + O	0.40 ± 0.08 [b]
Hx + O	-0.78 ± 0.08 [b]
Hx + E	-2.09 ± 0.10 [a]
Hx + G + E + O	-1.97 ± 0.03 [a]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 6-8 neonatal rats.

[a] p<0.01 when compared to Control [b] p<0.001 when compared to hypoxic group

Hypoxic rats- Hx, Hypoxic rats glucose treated - Hx+G, Hypoxic rats oxygen treated - Hx+O, Hypoxic rats glucose and oxygen treated - Hx+G+O, Hypoxic rats epinephrine treated – Hx + E, Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

Gene expression study of Phospholipase C using Real Time PCR analysis

The gene expression of phospholipase C mRNA showed a significant up regulation (p<0.001) in the hypoxic group compared to control. In Hx + G, Hx + G + O and Hx + O there was a significant reversal (p<0.001) of the expression to near control. Phospholipase C gene expression showed no significant reversal in Hx + E and Hx + G + E + O to near control.(Table. 2).

TABLE II
REAL TIME PCR AMPLIFICATION OF PHOSPHOLIPASE C MRNA FROM THE CORPUS STRIATUM OF CONTROL AND EXPERIMENTAL NEONATAL RATS

Experimental groups	Log RQ
Control	0
Hx	1.23 ± 0.13 [a]
Hx + G	0.12 ± 0.07 [b]
Hx + G + O	0.11 ± 0.04 [b]
Hx + O	0.68 ± 0.13 [b]
Hx + E	1.17 ± 0.12 [a]
Hx + G + E + O	1.02 ± 0.15 [a]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 6-8 neonatal rats.

[a] $p<0.01$ when compared to Control [b] $p<0.001$ when compared to hypoxic group

Hypoxic rats- Hx, Hypoxic rats glucose treated - Hx+G, Hypoxic rats oxygen treated - Hx+O, Hypoxic rats glucose and oxygen treated - Hx+G+O, Hypoxic rats epinephrine treated – Hx + E, Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

IV. DISCUSSION

Cyclic AMP is generated through the action of adenylyl cyclases, which is stimulated through appropriate G- protein coupled receptors (GPCRs) able to couple to the stimulatory guanine nucleotide regulatory protein, Gs [10]. cGMP synthesis is catalyzed by guanylate cyclase (GC), which converts GTP to cGMP. Membrane-bound GC is activated by peptide hormones such as the atrial natriuretic factor, while soluble GC is typically activated by nitric oxide to stimulate cGMP synthesis. cGMP is a common regulator of ion channel conductance, glycogenolysis, and cellular apoptosis. In our studies we observed an elevated cAMP and cGMP level in the corpus striatum of hypoxic neonatal rats. The imbalance in the redox system of oxidative phospholyration results in ROS production under hypoxic stress, which in turn activates the second messenger pathways as an adaptive modification. Millenab *et al* (2006) reported that the increased cAMP levels in hypoxia are due to the ERK-mediated autocrine generation of prostaglandin E2 [11]. Consistent with such a role for ERK, MEK inhibitors was found to normalize cAMP levels in hypoxic hPASM cells presumably by curtailing this autocrine response [11]. Resuscitation with glucose brought back the altered cAMP and cGMP level to near control due to the reduced ROS production. Resuscitation with 100% oxygen forms ROS which triggers cAMP and cGMP production. In epinephrine resuscitated groups the cAMP and cGMP levels are high as epinephrine triggers cAMP formation. Epinephrine acts as a α_2- and β-adrenoceptor agonist and α_2-adrenoceptors interact with β -adrenoceptors and vasopressin receptors for cAMP accumulation [12]. It was concluded that cAMP and cGMP play an important role in neonatal hypoxia, participate in the cellular signal transducation and promote the homeostatic response of the body to the stress.

IP3 functions by binding to the membrane-associated IP3 receptors (IP3R) [13]. Binding of IP3 to the receptor increases its sensitivity to Ca^{2+}, and only after Ca^{2+} is bound can trafficking of the Ca^{2+} into the cytosol take place. Notably, Ca^{2+} has a biphasic action on the IP3R with a stimulatory effect at low Ca2+ concentrations and an inhibitory effect at higher Ca^{2+} concentrations [14]. Acting as a signal transducer between two ubiquitous second messengers IP3 and Ca^{2+}, IP3R has been implicated in a variety of cellular and physiological processes as diverse as cell division, cell proliferation, apoptosis, fertilization, development, behaviour, memory and learning. In mammals, there are three distinct types of IP3R with splice variants observed among the types [15]. IP3-receptor is dominantly expressed in neuronal cells throughout the central nervous system [16]. Throughout the brain, the IP3R1 is the predominantly expressed member of the family and its mRNA is widely distributed.

|Our studies observed an elevated IP3 content in the striatum of hypoxic neonatal rats. The elevated IP3 level causes extra cellular release of Ca^{2+}, which in turn results in the activation of apoptotic pathways. Transfer of Ca^{2+} between intracellular stores and mitochondria provides physiological control of respiration. But this Ca^{2+} cycle also lead to cell death. If the matrix Ca^{2+} level rises too high, then deleterious changes in mitochondrial structure occur. In particular, mitochondria swell and rupture or undergo permeability transition, thereby releasing several pro-apoptotic factors into the cytoplasm, such as cytochrome C, second mitochondrial activator of caspases (SMAC/Diablo) or apoptosis-inducing factor (AIF) [17]. This leads to the generation of the 'apoptosome' and activation of caspases from inactive zymogens. It is well established that Ca^{2+} released through IP3 receptors is sequestered by mitochondria [18]. Furthermore, it has been demonstrated that the flow of Ca^{2+} specifically from IP3 receptors can cause mitochondrial permeability transition and activate the apoptotic cascade [19]. Alterations in phosphoinositide-mediated signal transduction lead to the loss of mAChR sensitivity, which is also observed in the present study. Glucose resuscitation, alone and along with oxygen effectively brought back the elevated IP3 level to near control. The intracellular glucose level acts as a regulator of IP3 formation and signaling.

Cyclic AMP response element binding (CREB) protein, a transcription factor, mediates responses to a number of physiological and pathological signals such as neurotransmitters, synaptic activity, depolarization, mitogens, hypoxia and other stress factors. In the present study the gene expression of CREB was down regulated in corpus striatum of hypoxic neonatal rats compared to control. Eventhough cAMP level was increased in hypoxic neonatal rats, the CREB expression declined. Adaptive response of the body to hypoxia activates the second messengers to encounter the stress. But acute and prolonged hypoxia triggers the cell death pathways by activating pro apoptotic genes like bax, bad and destabilizing jun- fos complex. The activation of apoptotic pathways down regulates the CREB expression thereby blocking the cAMP signaling cascade in hypoxic neonatal rats. Down regulation of CREB is a consequence of apoptotic pathway activation and down regulation of muscarinic receptor function. These findings suggest that

decreased CREB expression is the result of cell loss. The fact that CREB expression is known to be regulated in a number of systems [20] suggests that post-translational modification is not the only mechanism involved in the control of its trans-activation potential. Ca^{2+} increase induced by hyperosmotic stress promotes cell survival by recruiting CREB-mediated signaling. Resuscitation with glucose alone and along with oxygen reversed the down regulated CREB expression to near control. Hypoxic neonatal rats resuscitated with epinephrine or 100% oxygen did not show any reversal. Since glucose acts as immediate source of ATP, the hypoxic stress related ROS production and apoptosis is limited by administrating glucose to hypoxic neonatal rats.

In the present study, we observed hypoxia-mediated alterations in phospholipase C expression in the corpus striatum. Further we extended the studies to phospholipase C regulation with glucose, oxygen and epinephrine resuscitation for potential therapeutics which modulate signal transduction pathway for preventing CNS dysfunction in neonatal hypoxia. Our results showed an increased expression of phospholipase C in the corpus striatum of hypoxic neonatal rats when compared to control. We considered that the up regulation of the Phospholipase C in the brain regions of hypoxic neonatal rats contribute to the increased IP3 levels in hypoxic rats. Phospolipase C performs a catalytic mechanism, generating inositol triphosphate (IP3) and diacylglycerol (DAG). Altered phospholipase C expression fails to modulate the activity of down stream proteins important for cellular signaling. Defective expression of phospholipase C causes the impaired release of Ca^{2+} and brings down the level of intracellular calcium and thus failed to execute the normal neuronal function in the brain regions. During hypoxia fructose-1,6-bisphosphate initiates a series of neuroprotective signals which include PLC activation, small increases in [Ca^{2+}] and increased activity of the MEK/ERK signaling pathway [21] . Previous studies reports that phospholipase C-mediated signaling initiated by growth factor receptor types, are involved in long-term memory formation, a process that requires gene expression [22]. These evidences led us to propose that the enhancement of hypoxia-mediated phospholipase C gene expression could affect the central cognitive functions, which has been effectively protected by glucose resuscitation.

To summarize, an increased second messenger levels along with a down regulated expression of CREB and upregulated PLC gene was observed in the corpus striatum of neonates exposed to hypoxia. These points to the fact that even though the second messengers cAMP is activated as a stress response the signaling is not complete due to the absence of CREB expression. The efficient and timely supplementation of glucose reversed these alterations observed in hypoxia, 100% oxygen and epinephrine. Thus it is suggested from our studies that glucose administration immediately after hypoxia with oxygen as a resuscitation programme will be of tremendous advantage in neonatal care. Deeper understanding of mechanisms, through which hypoxia regulates the neurotransmitters and its signalling, could point towards the development of new therapeutic approaches to reduce or suppress the effects of hypoxia.

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