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Keywords (separated by '-')	$GABA_A$ - Hypoxia - Cerebral cortex - Brainstem - Bicuculline
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ORIGINAL RESEARCH

Decreased $GABA_A$ Receptors Functional Regulation in the Cerebral Cortex and Brainstem of Hypoxic Neonatal Rats: Effect of Glucose and Oxygen Supplementation

T. R. Anju · T. Peeyush Kumar · C. S. Paulose

Received: 15 June 2009 / Accepted: 11 December 2009

Abstract Hypoxia in neonates can lead to biochemical and molecular alterations mediated through changes in neurotransmitters resulting in permanent damage to brain. In this study, we evaluated the changes in the receptor status of $GABA_A$ in the cerebral cortex and brainstem of hypoxic neonatal rats and hypoxic rats supplemented with glucose and oxygen using binding assays and gene expression of $GABA_{A\alpha1}$ and $GABA_{A\gamma5}$. In the cerebral cortex and brainstem of hypoxic neonatal rats, a significant decrease in $GABA_A$ receptors was observed, which accounts for the respiratory inhibition. Hypoxic rats supplemented with glucose alone and with glucose and oxygen showed, respectively, a reversal of the $GABA_A$ receptors, and$GABA_{A\alpha1}$ and $GABA_{A\gamma5}$ gene expression to control. Glucose acts as an immediate energy source thereby reducing the ATP-depletion-induced increase in GABA and oxygenation, which helps in encountering anoxia. Resuscitation with oxygen alone was less effective in reversing the receptor alterations. Thus, the results of this study suggest that reduction in the $GABA_A$ receptors functional regulation during hypoxia plays an important role in mediating the brain damage. Glucose alone and glucose and oxygen supplementation to hypoxic neonatal rats helps in protecting the brain from severe hypoxic damage.

Keywords $GABA_A$ · Hypoxia · Cerebral cortex · Brainstem · Bicuculline

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Introduction

Hypoxic and hypoxic-ischemic (H-I) insults are predominant causes of injury in human fetuses and newborns and lead to extensive central nervous system damage (Finer et al. 1981; Levene et al. 1985; Myers 1972; Thornberg et al. 1995). Newborn babies are exposed to hypoxia and ischemia during the perinatal period as a result of stroke or problems with delivery or respiratory management after delivery (William et al. 2005). The fetal and newborn brain is particularly susceptible to hypoxia, which increases the risk for neurodevelopmental deficits, seizures, epilepsy, and life-span motor, behavioural, and cognitive disabilities. The brain is of special interest for hypoxia studies as it is extremely sensitive to reductions in oxygen supply. Hypoxia causes changes in brain neurotransmitters depending on its severity and duration. It has been suggested that an overabundance of excitatory synaptic inputs, excessive release of excitatory amino acids (EAAs) and subunit composition of EAA receptors that favors high Ca^{2+} conductance in the neonatal brain, contribute to vulnerability to H-I injury. Owing to known changes in GABA(γ-amino butyric acid)-ergic innervation during development, a strengthening of GABA-ergic input occurs resulting in an increase in their resistance to EAA toxicity (Tremblay et al. 1988; Stein and Vanucci 1988; Johnston 1995; Mishra et al. 2001).

Hypoxia in newborn infants results in severe life-long consequences and, hence, recognition of risk and knowledge of appropriate measures to treat fetal and neonatal hypoxia and hypoxemia are of utmost importance in neonatal care. The traditional resuscitation of newborn infants, who are asphyxiated at birth, was practiced with administration of 100% oxygen and intravenous fluids which include 10% glucose. However, there has been a

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considerable concern over the risks of using 100% oxygen, which is reported to cause glutamate-mediated neurotoxicity (Paulose et al. 2007) and free radical-mediated damage to the brain (Anju et al. 2009). The functional regulation of brain neurotransmitters in the ventilatory response during neonatal hypoxia and various resuscitation methods play an important role in proper management of brain damage due to hypoxia.

The ventilatory response to hypoxia is influenced by the balance between inhibitory (GABA, glycine, and taurine) and excitatory (glutamate and aspartate) amino acid neurotransmitters. GABA and glutamate are the two important neurotransmitters involved in hypoxic ventilatory response. GABA in the nucleus tractus solitarii has a pivotal role in the hypoxic ventilatory decline (HVD), and this mechanism is not activated without chemoreceptor stimulation (Tabata et al. 2001).

Anoxia-tolerant vertebrates decrease their metabolic rate by 70% or more during anoxia, with an increase in concentration of GABA (Göran 1992). Long-term hypoxia produces a significant but reversible reduction on GABA binding to $GABA_A$ receptor sites in cerebral cortex, which reflect an adaptive response to this sustained pathophysiological state (Viapiano et al. 2001). Hypoxia has been a selective pressure in conserving GABA and glutamate as major inhibitory and excitatory neurotransmitters in vertebrates as well as invertebrates (Nilsson and Lutz 1993).

This study aims at investigating the role of GABA in the ventilatory response to hypoxia by studying the receptor kinetics of $GABA_A$ receptors and gene expression of $GABA_{A\alpha1}$ and $GABA_{A\gamma5}$ in the cerebral cortex and brainstem of hypoxia-induced neonatal rats. We also investigated the role of glucose and oxygen supplementation in regulating the $GABA_A$ receptor subtypes in hypoxia. This study helps to understand the $GABA_A$ receptor regulation of the ventilatory response to neonatal hypoxia and establishes the effectiveness of glucose and oxygen resuscitation programme.

Materials and Methods

Chemicals Used for the Study

Bicuculline methoiodide, Tris buffer and Tri-reagent kit used in this study were purchased from SIGMA Chemical Co., St. Louis, USA. Bicuculline methyl chloride (−)-[methyl-^{3}H] (Specific activity—82.9 Ci/mmol) was purchased from NEN Life Sciences Products, Inc., Boston USA. ABI PRISM High Capacity cDNA Archive kit, Primers and Taqman probe for Real-Time PCR were purchased from Applied Biosystems, FosterCity, CA, USA. Taqman probes used were $GABA_{A\alpha1}$ (Rn 00788315) and $GABA_{A\gamma5}$ (Rn 00577639).

Induction of Hypoxia in Neonatal Rats

Wistar neonatal rats of 4 days old weighing 6.0–7.5 g were used for the experiments. Induction of hypoxia and supplementation of glucose and oxygen were done according to the procedure of Paulose et al. (2007). Experimental animals were grouped 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 (ip) immediately after induction of hypoxia (Hx + G); (iv) hypoxic neonatal rats were supplied with 100% oxygen for 30 min immediately after induction of hypoxia (Hx + O); (v) hypoxic neonatal rats were injected 10% dextrose (500 mg/kg body wt) ip immediately after induction of hypoxia and then treated with 100% oxygen for 30 min (Hx + G + O). Each experiment was carried out with 6–8 rats from each group. All the experiments were carried out at room temperature. All the groups of neonatal rats were maintained under optimal conditions, 12 h light and 12 h dark. Rats were weighed and sacrificed by decapitation. The cerebral cortex and brainstem were dissected out quickly over ice according to the procedure of Glowinski and Iversen (1966), and the tissues were stored at −80°C for all the experiments. All animal care and procedures were in accordance with Institutional and National Institute of Health guidelines, and care was taken to minimize the suffering of the experimental rats.

$GABA_A$ Receptor-Binding Assays in the Cerebral Cortex and Brainstem

[^{3}H]bicuculline binding to the $GABA_A$ receptor was assayed in Triton X-100-treated synaptic membranes (Kurioka et al. 1981). Crude synaptic membranes were prepared using sodium-free 10 mM tris buffer, pH 7.4. Each assay tube contained a protein concentration of 0.1–0.2 mg. In saturation-binding experiments, 5–40 nM concentrations of [^{3}H]bicuculline was incubated with and without excess of unlabeled bicuculline (100 μM), and in competition binding experiments, the incubation mixture contained 30nM of [^{3}H]bicuculline with and without bicuculline at a concentration range of 10^{-8}M to 10^{-4}M were used. The incubation was continued for 20 min at 0–4°C and terminated by centrifugation at 35,000×g for 20 min. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

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Protein Extraction and Determination of Concentration

Cerebral cortex and brainstem were homogenized in a polytron homogenizer with 20 volumes of cold 10 mM Tris buffer, pH 7.4, and the crude synaptic membrane obtained by the method of Kurioka et al. (1981) was resuspended in appropriate volumes of buffer. Protein was measured by the method of Lowry et al. (1951) with BSA as standard.

Receptor-Binding Data Analysis

Linear Regression Analysis for Scatchard Plots

The receptor-binding parameters were determined using Scatchard analysis (1949). The specific binding was determined by subtracting non-specific binding from the total binding. 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.

Nonlinear Regression Analysis for Displacement Curve

Competitive binding data were analyzed using nonlinear regression curve-fitting procedure (GraphPad PRISM™, San Diego, USA). The data of the competitive binding assays were represented graphically with the log of concentration of the competing drug on *x*-axis and percentage of the radioligand bound on the *y*-axis. The steepness of the binding curve can be quantified with a slope factor, often called a Hill slope. A one-site competitive binding curve that follows the law of mass action has a slope of 1.0, and a two-site competitive binding curve has a slope less than 1.0. The concentration of competitor that competes for half the specific binding was defined as EC_{50}, which is same as IC_{50}. The affinity of the receptor for the competing drug is designated as K_i and is defined as the concentration of the competing ligand that binds to half the binding sites at equilibrium in the absence of radioligand or other competitors (Cheng and Prusoff 1973).

Analysis of Gene Expression by Real-Time PCR

RNA was isolated from the cerebral cortex and brainstem using Tri reagent. Total cDNA synthesis was performed using ABI PRISM cDNA Archive kit. Real-Time PCR assays were performed in 96-well plates in an ABI 7300 Real-Time PCR instrument (Applied Biosystems, Foster City, CA, USA). PCR analyses were conducted with gene-specific primers, and fluorescently labeled Taq probe for $GABA_{A\alpha1}$ (Rn 00788315) and $GABA_{A\gamma5}$ (Rn 00577639) designed by Applied Biosystems. Endogenous control (β-actin) labeled with a reporter dye was used as internal control. All the reagents were purchased from Applied Biosystems. The real-time data were analyzed with Sequence Detection Systems software version 1.7. All the reactions were performed in duplicate.

The ΔΔCT method of relative quantification was used to determine the fold change in expression. This was done by first 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 ($2^{-\Delta\Delta CT}$).

Statistical Analysis

The equality of all the groups was tested by the analysis of variance (ANOVA) technique for different values of *p*. Further, the pairwise 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.

Results

$GABA_A$ Receptor Function in Cerebral Cortex and Brainstem of Hypoxic Neonatal Rats

Binding studies of [^{3}H] bicuculline against bicuculline in cerebral cortex of hypoxic neonatal rats showed a significant decrease in B_{max} ($P < 0.001$) with a significant increase in K_d ($P < 0.001$) compared to control. This reflected a decreased receptor number with low affinity in the cerebral cortex of hypoxic neonatal rats compared to control. In brainstem, binding studies showed a significant decrease in B_{max} ($P < 0.05$) without any significant change in K_d compared to control. This showed a decreased receptor number for $GABA_A$ receptors in the brainstem of hypoxic neonates in the brain stem (Table 1).The binding data were confirmed by competition binding assay with [^{3}H] bicuculline against different concentrations of bicuculline. $GABA_A$ affinity in the cerebral cortex and brainstem of control and hypoxic neonatal rats fitted to a two-site model with Hill slope value away from unity (Figs. 1, 2).

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Table 1 [^{3}H] bicuculline-binding parameters in the cerebral cortex and brain stem of control and experimental groups of neonatal rats

Condition	Cerebral cortex		Brain stem	
	B_{max} (fmoles/mg protein)	K_d (nM)	B_{max} (fmoles/mg protein)	K_d (nM)
Control	182.50 ± 0.63	5.21 ± 0.085	38.95 ± 0.05	1.30 ± 0.05
Hx	143.33 ± 0.33[a]	8.42 ± 0.165[a]	23.45 ± 0.25[a]	1.40 ± 0.10
Hx + O	130.00 ± 0.29[a]	8.09 ± 0.09[a]	40.01 ± 0.23[c,d]	3.12 ± 0.52[a,d]
Hx + G	161.67 ± 0.22[b,e]	8.627 ± 0.17[a]	39.10 ± 0.22[d]	3.25 ± 0.15[a,d]
Hx + G + O	159.17 ± .065[c,e]	5.49 ± 0.61[d]	35.00 ± 0.31[b,d]	1.61 ± 0.05[a,e]

Values are mean ± SEM of 4–6 separate experiments. Each group consists of 6–8 rats

[a] $P < 0.001$, [b] $P < 0.01$, [c] $P < 0.05$ when compared with control

[d] $P < 0.001$, [e] $P < 0.05$ when compared with hypoxic group

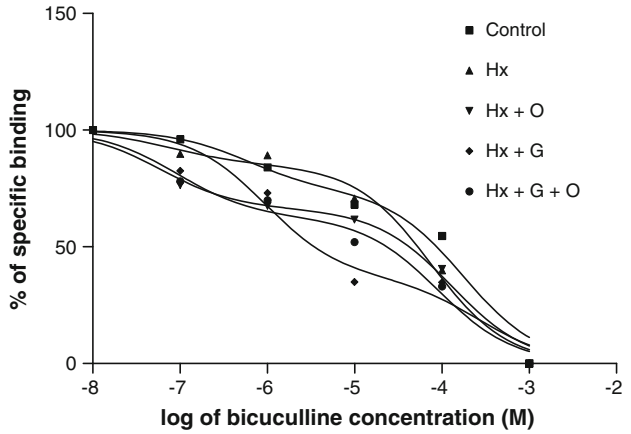

Fig. 1 Binding parameters of [^{3}H] bicuculline against bicuculline in the cerebral cortex of experimental neonatal rats

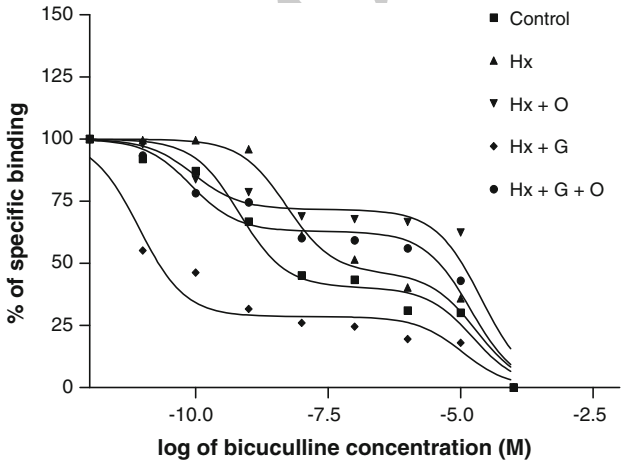

Fig. 2 Binding parameters of [^{3}H] bicuculline against bicuculline in the brain stem of experimental neonatal rats

$GABA_A$ Receptor Function in Cerebral Cortex and Brainstem of Glucose and Oxygen Supplemented Groups

In the cerebral cortex of Hx + G, a significant increase in B_{max} ($P < 0.01$) compared to the hypoxic group was observed, showing a reversal of receptor number to near control. In Hx + G + O, B_{max} reversed to near control level with a high affinity. In Hx + O, a significant decrease in B_{max} ($P < 0.001$) with a significant increase in K_d ($P < 0.001$) was observed compared to control, showing a low affinity toward $GABA_A$ receptors in oxygen supplemented group (Table 1). Competitive binding assay with [^{3}H] bicuculline against different concentrations of bicuculline showed that $GABA_A$ affinity in the cerebral cortex of Hx + O, Hx + G and Hx + G + O fitted to a two-site model with Hill slope value away from unity. The gene expression studies by real-time PCR analysis showed that in cerebral cortex, $GABA_{A\alpha1}$ and $GABA_{A\gamma5}$ receptor mRNA was significantly down regulated in Hx ($P < 0.001$). Glucose treatment to hypoxic (Hx + G, Hx + G + O) significantly ($P < 0.001$) reduced the down regulation compared to hypoxic groups. Hx + O showed significant ($P < 0.001$) down regulation compared to both control and hypoxic groups (Table 2, Figs. 3, 4).

In the brainstem of glucose supplemented group (Hx + G), B_{max} showed a reversal to near control. In Hx + G + O, B_{max} and K_d were reversed to near control. In the oxygen-supplemented group (Hx + O), a significant increase in B_{max} ($P < 0.05$) with a significant increase in K_d ($P < 0.001$) compared to control was observed. This showed an increase in receptor number with less affinity in the oxygen-supplemented group. (Table 1). $GABA_A$ affinity in the brainstem of Hx + O, Hx + G, and Hx + G + O fitted to a two-site model with Hill slope value away from unity. The gene expression studies by real-time PCR analysis showed that $GABA_{A\alpha1}$ and $GABA_{A\gamma5}$ receptor mRNA was significantly down regulated in Hx ($P < 0.001$). Glucose and oxygen supplementation to hypoxic (Hx + G, Hx + O, and Hx + G + O) showed an up regulation to near control (Table 3, Figs. 5, 6).

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Table 2 Real-Time amplification of GABA (A)α_1 and GABA (A)$_{\gamma 5}$ receptor mRNA from the cerebral cortex of control and experimental rats

Animal status	Log RQ value	
	$GABA_A\alpha_1$	$GABA_{A\gamma 5}$
Control	0	0
Hx	-0.25 ± 0.02^{a}	-1.97 ± 0.20^{b}
Hx + O	$-0.27 \pm 0.06^{a,c}$	$-1.13 \pm 0.04^{b,d}$
Hx + G	$-0.06 \pm 0.03^{a,c}$	$-0.32 \pm 0.13^{b,d}$
Hx + G + O	$-0.07 \pm 0.11^{a,c}$	$-1.19 \pm 0.03^{b,d}$

Values are mean ± SD of 4–6 separate experiments. Each group consist of 6–8 rats

[a] $P < 0.05$, [b] $P < 0.001$ when compared to control, [c] $P < 0.05$, [d] $P < 0.001$ when compared to hypoxic group

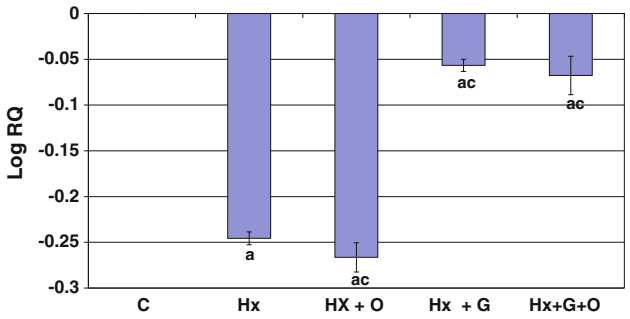

Fig. 3 Real Time amplification of GABA (A)α_1 receptor mRNA from the Cerebral cortex of control and experimental neonatal rats. *Note*: Values are mean ± SD of 4–6 separate experiments. Each group consist of 6–8 rats. [a] $P < 0.05$ when compared to control, [c] $P < 0.05$ when compared to hypoxic group

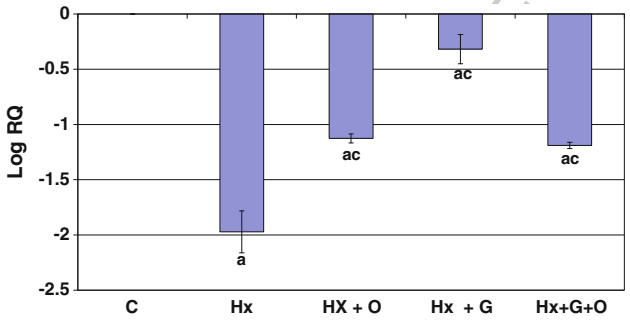

Fig. 4 Real-Time amplification of GABA (A)γ_5 receptor mRNA from the Cerebral cortex of control and experimental neonatal rats. *Note*: Values are mean ± SD of 4–6 separate experiments. Each group consists of 6–8 rats. [a] $P < 0.05$ when compared to control, [c] $P < 0.05$ when compared to hypoxic group

Table 3 Real-Time amplification of GABA (A)α_1 and GABA (A)$_{\gamma 5}$ receptor mRNA from the brain stem of control and experimental rats

Animal status	Log RQ value	
	$GABA_A\alpha_1$	$GABA_{A\gamma 5}$
Control	0	0
Hx	-0.23 ± 0.03^{a}	-0.44 ± 0.03^{b}
Hx + O	$0.04 \pm 0.01^{a,c}$	$0.42 \pm 0.01^{b,d}$
Hx + G	$0.03 \pm 0.01^{a,c}$	$0.32 \pm 0.01^{b,d}$
Hx + G + O	$0.01 \pm 0.02^{a,c}$	$0.27 \pm 0.02^{b,d}$

Values are mean ± SD of 4–6 separate experiments. Each group consist of 6–8 rats

[a] $P < 0.05$, [b] $P < 0.001$ when compared to control, [c] $P < 0.05$, [d] $P < 0.001$ when compared to hypoxic group

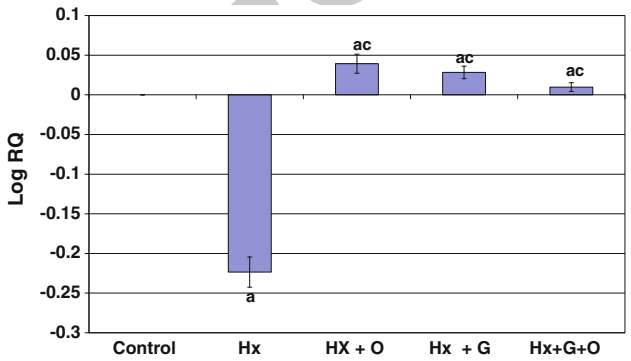

Fig. 5 Real Time amplification of GABA (A)α_1 receptor mRNA from the brain stem of control and experimental neonatal rats. *Note*: Values are mean ± SD of 4–6 separate experiments. Each group consists of 6–8 rats. [a] $P < 0.05$ when compared to control, [c] $P < 0.05$ when compared to hypoxic group

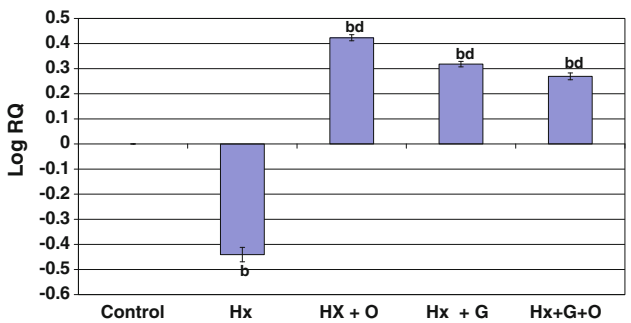

Fig. 6 Real-Time amplification of GABA (A)γ_5 receptor mRNA from the Brain stem of control and experimental neonatal rats. *Note*: Values are mean ± SD of 4–6 separate experiments. Each group consists of 6–8 rats. [b] $P < 0.001$ when compared to control, [d] $P < 0.001$ when compared to hypoxic group

Discussion

In this study, we investigated the functional regulation of $GABA_A$ receptors in hypoxic neonatal rats and the role of glucose and oxygen in altering the receptor status. Hypoxia has profound cellular effects mediated by altered activity and expression of proteins (Bandyopadhyay et al. 1999). These alterations prepare the cell to cope with the HVD

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and reduction in energy supply to vital organs through various mechanisms. Survival in low-oxygen environments requires adaptation of sympathorespiratory control networks located in the brainstem. One of the major adaptations to overcome low-oxygen tension is a change in the levels of various neurotransmitters and its receptors for mediating the mechanisms to maintain body homeostasis. GABA and glutamate are the most important neurotransmitters involved in the ventilatory response to hypoxic condition. With acute hypoxia, there is a biphasic ventilatory response with an initial hyperventilation followed by a fall in ventilation to values above those in the pre-hypoxic level. Central neurotransmitters are essential in this response (Homayoun 2006).

It is reported that hypothermic newborn piglets have a depressed ventilatory response to hypoxia due to an increase in cental nervous system GABA levels (Qiming xiao et al. 2000). Infusion of the GABA antagonist bicuculline caused augmentation of the hyperventilatory response to acute hypoxia (Homayoun 2006). Sustained hypoxia has been associated with increased GABA formation, which is inhibitory to respiration. During sustained or severe hypoxia, the level of alpha ketoglutarate, which is important in the degradation of GABA and synthesis of glutamate, declines rapidly due to blockage of aerobic metabolism. With a reduction in alpha ketoglutarate, GABA cannot be degraded. However, it can still be formed from glutamate by the action of the anaerobic enzyme glutamic acid decarboxylase, which allows GABA formation to proceed during hypoxia. Decreased $GABA_A$ receptors observed in the cerebral cortex and brain stem of hypoxic neonates may increase the GABA level in the brain regions, thereby augmenting the severity of HVD.

$GABA_A$ receptors mediate the majority of fast inhibitory synaptic interactions in the mammalian brain. In the adult brain, networks of neurons containing $GABA_A$-ergic receptors have been implicated in the maintenance of rhythmic activities of neuronal circuits (Whittington et al. 1995; Wang and Buzsaki 1996) and the precise control of the timing of excitability in individual neurons (Tsubokawa and Ross 1996). GABA neurotransmission serve both excitatory and inhibitory roles during early development (Chen et al. 1996). Subunit diversity appears to underlie distinctive roles for $GABA_A$ receptors in the development of the nervous system (Laurie et al. 1992; Poulter et al. 1992; Ma et al. 1993).

We observed a significant decrease in B_{max} of $GABA_A$ receptors in both cerebral cortex and brainstem of hypoxic neonatal rats. Central nervous system is severely affected by hypoxic–ischemic insults during the prenatal–perinatal period, including imbalance in excitatory and inhibitory neurotransmitter release (Gil et al. 2004). Hypoxia increases GABA levels in neurons by ATP depletion-induced activation of glutamate decarboxylase and by inhibiting GABA transaminase. GABA levels were highly correlated with endogenous glutamate levels. Hypoxia increased GABA concentrations primarily in neurons and their processes. Severe hypoxic ATP depletion increased the release of both GABA and glutamate (Madl and Royer 2000). It is reported that prolonged exposure to hypobaric hypoxia transiently reduces $GABA_A$ receptor number in mice cerebral cortex (Viapiano et al. 2001).

In this study, we observed a significant decrease in the $GABA_A$ receptor number with a decreased affinity in the cerebral cortex of hypoxic neonates. Even though the GABA level is increased under hypoxic condition, $GABA_A$ receptor number is less than that of control. The decreased receptor number, in turn, results in further accumulation of GABA due to the blockage of GABA degrading pathways resulting in HVD. In brainstem also, the receptor number showed a significant decrease compared to control which greatly affects the respiratory control networks to encounter oxygen deprivation to tissues. We suggested that an up regulation of GABA receptor will help in overcoming the ventilatory decline during hypoxic condition.

In our study, supplementation of glucose alone and glucose along with 100% oxygen to hypoxic neonates showed a reversal in the receptor number to near control in the cerebral cortex and brainstem. The combination of glucose and oxygen was found to be the most effective resuscitation method. Glucose is supplemented during hypoxia to provide an immediate resuscitation to the stress condition by acting as an instant source of energy to the brain. Hattori and Wasterlain (2004) observed a reduction in the blood glucose levels and substantially increased cerebral glucose utilization (Vannucci and Hagberg 2004) as a result of hypoxic stress in experimental rats. We observed that supplementation of glucose is effective in increasing the $GABA_A$ receptor status, thereby decreasing the GABA level in the cortex and brainstem. Since glucose provides an immediate and instant energy to tissues, it helps in encountering the ATP depletion-induced increase in GABA levels and, hence, the inhibition of respiration. Ito et al. (1994) observed a dose-dependent reduction in the cerebral glucose utilization after intravenous administration of various doses of muscimol, an agonist of $GABA_A$. A linear relationship was observed between the $GABA_A$ receptor occupancy of muscimol and the decrease in the cerebral glucose utilization (Ito et al. 1994). Bailey et al. (2007) reported that glucose dose-dependently increased the expression of $GABA_A$ receptor subunits in pancreatic cells.

One of the routine methods of resuscitation for severe hypoxia is the immediate administration of oxygen. We observed that 100% oxygen supplementation for neonatal hypoxia is not as effective as the combination of glucose

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and oxygen or administration of glucose alone. In the cerebral cortex of Hx + O, $GABA_A$ receptors showed a significant decrease, even below the hypoxic level. In the brainstem, even though oxygen supplementation showed an increased receptor number, the receptor affinity for the ligand is found to be very less. Thus, the receptor and gene expression studies of $GABA_A$ showed that administration of 100% oxygen to hypoxic neonates did not bring down the GABA level to encounter HVD. The 100% of oxygen generated abnormally high levels of reactive oxygen species (ROS), which cause dysfunction of defensive antioxidant system of cells by altering enzyme activity (Bandyopadhyay et al. 1999; Anju et al. 2009) and act as a factor for neurodegeneration (Matharan et al. 2004). Hypoxemic piglets resuscitated with 100% O_2 also showed increased cerebral injury, cortical damage, and early neurologic disorders (Temesvari et al. 2001; Munkeby et al. 2004; Shimabuku et al. 2005). Based on behavioral studies and the studies on acetylcholinesterase, Finla et al. (2008) reported the efficiency of glucose and combination of glucose and oxygen resuscitation methods, and the damaging effects of oxygen supplementation alone. The reduction in $GABA_A$ receptor number or receptor affinity in the cortex and brainstem during oxygen supplementation is suggested to be due to tissue damage caused by the formation of free radicals or ROS.

In order to summarize the findings of this study, $GABA_A$ receptors were found to be significantly reduced in the cortex and brainstem of hypoxic neonatal rats. The change in the receptor status observed under hypoxic condition was reversed to control level by the supplementation of 10% glucose alone and combination of glucose and oxygen. The administration of 100% oxygen alone showed significantly reduced receptor level near to hypoxic level, which shows the damaging effects of resuscitation with oxygen alone. Our results point out the importance of $GABA_A$ receptors in controlling the ventilatory response during hypoxic insult and also the positive effects of glucose and combination of glucose and oxygen supplementation on hypoxia in neonates. The timely resuscitation with glucose or glucose and oxygen will help to increase the ventilatory response and to reduce the brain damage due to hypoxia. This has clinical significance in neonatal care and healthy intellect during later developmental period. Further studies with the experimental rats, at different timings after hypoxic exposure, will show the extent of brain damage for initiating corrective measures at the molecular level.

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References

Anju TR, Athira B, Paulose CS (2009) Superoxide dismutase functional regulation in neonatal hypoxia: effect of glucose, oxygen and epinephrine. Indian J Biochem Biophys 46:166–171

Bailey SJ, Ravier MA, Rutter GA (2007) Glucose-dependent regulation of gamma aminobutyric acid ($GABA_A$) receptor expression in mouse pancreatic islet-cells. Diabetes 56:320–327

Bandyopadhyay U, Das D, Ranajit K, Banerjee V (1999) Reactive oxygen species: oxidative damage and pathogenesis. Curr Sci 77:658–666

Cheng Y, Prusoff WH (1973) Relationship between the inhibition constant and the concentration of an inhibitor that causes a 50% inhibition of an enzymatic reaction. Biochem Pharmacol 22:3099–3108

Finer NN, Robertson C, Richards RT, Pinnell LE, Peter KL (1981) Hypoxic-ischemic encephalopathy in term neonates: perinatal factors and outcome. J Pediatr 98:112–117

Finla C, Amee K, Paulose CS (2008) Acetylcholine esterase activity and behavioral response in hypoxia induced neonatal rats: effect of glucose, oxygen and epinephrine supplementation. Brain Cogn 68:59–66

Gil DJR, Carmona C, Negri G, Fiszer de Plazas S (2004) Hypoxia differentially reduces $GABA_A$ receptor density during embryonic chick optic lobe development. Neurochem Res 29:681–686

Glowinski J, Iversen LL (1966) Regional studies of catecholamines in the rat brain: the disposition of [^{3}H] Norepinephrine, [^{3}H] DOPA in various regions of…the brain. J Neurochem 13:655–669

Göran EN (1992) Evidence for a role of GABA in metabolic depression during anoxia in crucian carp (carassius carassius). J Exp Biol 164:243–259

Hattori H, Wasterlain CG (2004) Posthypoxic glucose supplement reduces hypoxic–ischemic brain damage in the neonatal rat. Ann Neurol 28:122–128

Homayoun K (2006) Midbrain neurotransmitters in acute hypoxic ventilatory response. Adv Exp Med Biol 580:223–226

Ito K, Sawada Y, Sugiyama Y, Suzuki H, Hanano M, Iga T (1994) Linear relationship between $GABA_A$ receptor occupancy of muscimol and glucose metabolic response in the conscious mouse brain. Clinical implication based on comparison with benzodiazepine receptor agonist. Drug Metab Dispos 22:50–54

Johnston MV (1995) Neurotransmitter and vulnerability of the developing brain. Brain Dev 17:301–306

Levene MI, Kornberg J, Williams THC (1985) The incidence and severity of post-asphyxial encephalopathy in full term infants. Early Human Devel 11:21–26

Lowry OH, Rosebrough NJ, Farr AL, Randall J (1951) Protein measurement with folin phenol reagent. J Biol Chem 193:265–275

Madl JE, Royer SM (2000) Glutamate dependence of GABA levels in neurons of hypoxic and hypoglycemic rat hippocampal slices. Neuroscience 96:657–664

Matharan TS, Laemmel E, Duranteau J, Vicaut E (2004) After hypoxia and glucose depletion causes reactive oxygen species production by mitochondria in HUVEC. Am J Physiol Regul Integr Comp Physiol 287:R1037–R1043

Mishra OP, Fritz KI, Delivoria-Papadopoulos M (2001) NMDA receptor and neonatal hypoxic brain injury. Ment Ret and Dev Disabil Res Rev 7:249–253

Munkeby BH, Borke WB, Bjornland K, Sikkeland LL, Borge GL, Halvorsen BH (2004) Resuscitation with 100% O_2 increases cerebral injury in hypoxemic piglets. Pediatr Res 56:783–790

Myers RE (1972) Two patterns of perinatal brain damage and their conditions of occurrence. Am J Obstet Gynecol 112:246–276

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MS Code : CEMN-656 CP DISK

Nilsson GE, Lutz PL (1993) Role of GABA in hypoxia tolerance, metabolic depression and hibernation-possible links to neurotransmitter evolution. Comp Biochem Physiol C Comp Pharmacol Toxicol 105:329–336

Paulose CS, Finla C, Reas KS, Amee K (2007) Neuroprotective role of Bacopa monnieri extract in epilepsy and effect of glucose supplementation during hypoxia:Glutamate receptor gene expression. Neurochem Res 33:1663–1671

Scatchard G (1949) The attractions of proteins for small molecules and ions. Ann NY Acad Sci 51:660–672

Shimabuku R, Ota A, Pereyra S, Veliz B, Paz E, Nakachi G (2005) Hyperoxia with 100% oxygen following hypoxia–ischemia increases brain damage in newborn rats. Biol Neonate 88: 168–171

Stein DT, Vanucci RC (1988) Calcium accumulation during the evolution of hypoxic-ischemic brain damage in the immature rat. J Cereb Blood Flow Metab 8:834–842

Susumu K, Kimura T, Matsuda M (2006) Effects of sodium and bicarbonate ions on gamma amino butyric acid receptor binding in synaptic membranes of rat brain. J Neurochem 37:418–421

Tabata M, Kurosawa H, Kikuchi Y, Hida W, Ogawa H, Okabe S, Tun Y, Hattori T, Shirato K (2001) Role of GABA within the nucleus tractus solitarii in the hypoxic ventilatory decline of awake rats. Am J Physiol Regul Integr Comp Physiol 281:R1411–R1419

Temesvari P, Karg E, Bódi I, Németh I, Pintér S, Lazics K (2001) Impaired early neurologic outcome in newborn piglets reoxygenated with 100% oxygen compared with room air after pneumothorax-induced asphyxia. Pediatr Res 49:812–819

Thornberg E, Thiringer K, Odeback A, Milsom I (1995) Birth asphyxia: incidence, clinical course and outcome in Swedish population. Acta Paediatr 84:927–932

Tremblay E, Roisin MP, Represa A, Charriaut-Marlangue C, Ben-Ari Y (1988) Transient increased density of NMDA-binding sites in the developing rat hippocampus. Brain Res 461:393–396

Vannucci SJ, Hagberg H (2004) Hypoxia–ischemia in the immature brain. J Exp Biol 207:3149–3154

Viapiano MS, Mitridate de Novarab AM, Fiszer de Plazasb S, Bozzinic CE (2001) Prolonged exposure to hypobaric hypoxia transiently reduces $GABA_A$ receptor number in mice cerebral cortex. Brain Res 894:31–36

Xiao Q, Suguihara C, Hehre D, Devia C, Huang J, Bancalari E (2000) Effects of GABA receptor blockade on the ventilatory response to hypoxia in hypothermic newborn piglets. Pediatr Res 47: 663–668

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