Published in final edited form as:

Adv Exp Med Biol. 2015 ; 860: 1–8. doi:10.1007/978-3-319-18440-1_1.

Epigenetic regulation of Carotid Body Oxygen Sensing: Clinical Implications

Jayasri Nanduri and Nanduri R. Prabhakar

Jayasri Nanduri: nanduri@uchicago.edu; Nanduri R. Prabhakar: jnanduri@uchicago.edu

Abstract

Recurrent apnea with intermittent hypoxia (IH) is a major clinical problem in infants born preterm. Recent epidemiological studies showed that adults who were born preterm exhibit increased incidence of sleep-disordered breathing and hypertension. Thus, apnea of prematurity predisposes individuals to autonomic dysfunction in adulthood. Experimental studies showed that adult rats exposed to IH as neonates exhibit augmented carotid body and adrenal chromaffin cells (AMC) response to hypoxia and irregular breathing with apneas and hypertension. The enhanced hypoxic sensitivity of the carotid body and AMC in adult rats exposed to neonatal IH was associated with increased oxidative stress, decreased expression of genes encoding anti-oxidant enzymes, and increased expression of pro-oxidant enzymes. Epigenetic mechanisms including DNA methylation leads to long-term changes in gene expression. The decreased expression of the *Sod2* gene, which encodes the anti-oxidant enzyme, superoxide dismutase 2, was associated with DNA hypermethylation of a single CpG dinucleotide close to the transcription start site. Treating neonatal rats with decitabine, an inhibitor of DNA methylation, during IH exposure prevented the oxidative stress, enhanced hypoxic sensitivity, and autonomic dysfunction in adult rats. These findings suggest that epigenetic mechanisms, especially DNA methylation contributes to neonatal programming of hypoxic sensitivity and the ensuing autonomic dysfunction in adulthood.

Keywords

O₂ sensing; apnea; epigenetic mechanisms; DNA hypermethylation; exocytosis; oxidative stress

1.1 Introduction

Recurrent apnea (i.e., brief cessation of breathing) with intermittent hypoxia (IH) is a major clinical problem affecting a substantial population of infants born preterm (Nock *et al*, 2004; Poets *et al*, 1994). Infants with apnea of prematurity exhibit autonomic dysfunction manifested by clinical signs of increased sympathetic nerve activity (Lagercrantz *et al*, 1990), defective sympatho-adrenal function (Lagercrantz & Sjöquist, 1980) and augmented ventilatory response to hypoxia (Nock *et al*, 2004). These co-morbidities are attributed to augmented chemosensory reflex arising from the carotid body, the principal sensory organ for monitoring arterial blood O₂ levels (Kumar & Prabhakar, 2012). Recent studies reported

augmented hypoxic sensory response of the carotid body in neonatal rats exposed to IH (Pawar *et al*, 2008). Increased catecholamine (CA) secretion from adrenal medullary chromaffin cells (AMC) is another important homeostatic mechanism for preserving cardiovascular function in neonates under hypoxia (Lagercrantz & Bistoletti, 1977; Seidler & Slotkin, 1985). IH also enhances hypoxia-evoked CA secretion from AMC in neonatal rats (Souvannakitti *et al*, 2009a). Remarkably, the effects of neonatal IH on the carotid body and AMC persist into adult life (Pawar *et al*, 2008; Souvannakitti *et al*, 2009b). While these findings demonstrate that neonatal IH leads to long-lasting changes in O₂ sensing by the carotid body and AMC, little is known about the underlying molecular mechanisms.

It is being increasingly appreciated that epigenetic mechanisms, especially during development, lead to long-term changes in gene expression, which have been implicated in adult diseases resulting from perturbations in fetal/neonatal period (Feinberg, 2007). Classic examples include association of low birth weight to risk for developing adult onset of cardiovascular disease (Barker *et al*, 1993) and type 2 diabetes (Ravelli *et al*, 1998). Epigenetic changes are heritable modifications of DNA that do not involve changes in DNA primary sequence. Three mechanisms contribute to epigenetic modifications: a) silencing RNAs, b) DNA methylation and c) histone modifications (Feinberg, 2007), which either singularly or conjointly determine whether a gene is activated or silenced. Of the three mechanisms, DNA methylation is a key mechanism mediating fetal/neonatal-based adult disease (Niculescu *et al*, 2005; Waterland & Jirtle, 2003). This article describes recent studies showing the involvement of epigenetic mechanisms, especially DNA methylation, in the neonatal programming of O₂ sensing by the carotid body and AMC and its impact on cardio-respiratory homeostasis.

1.2. Neonatal IH augments carotid body and AMC responses to hypoxia

Neonatal rat pups are neurologically immature and resemble prematurely born human infants. The effects of IH on the carotid body responses to hypoxia were examined in neonatal rats. Rat pups with the dams were exposed to IH (15s of hypoxia followed by 5 min of normoxia, 9 episodes/h, eight hours/day) from ages P0–P10 (Pawar *et al*, 2009). During each episode of IH, blood O₂ saturation decreased from 98 to 82 % (Nanduri *et al*, 2012). Carotid bodies from control pups reared under normoxia responded poorly to hypoxia, and the response was relatively slow in onset. In striking contrast, carotid bodies from IH exposed pups exhibited robust sensory response to hypoxia, much similar to the adult carotid bodies, and the response was fast occurred within few seconds after the onset of hypoxia (Peng *et al*, 2004; Pawar *et al*, 2008; Fig. 1)

A number of studies reported that hypoxia by directly acting on AMC evokes CA secretion in neonatal rats (Lagercrantz & Bistoletti, 1977; Seidler & Slotkin, 1985; Takeuchi *et al*, 2001; Thompson *et al*, 1997). In IH exposed neonatal rats, the number of AMC responding to hypoxia and the magnitude of CA secretion for a given level of hypoxia were greater than control rat pups (Souvannakitti *et al*, 2009b). Further analysis showed that the increased CA secretion by hypoxia was due to more number of secretory events as well as the amount of CA released per secretory event. In addition, IH also increased both norepinephrine and epinephrine contents in neonatal adrenal medullae. In striking contrast, hypoxia-evoked CA

secretion was reduced in rat pups exposed to continuous hypobaric hypoxia (0.4 ATM), suggesting that the augmented secretory response of AMC is unique to IH (Souvannakitti *et al*, 2009a and b; Fig. 2). These findings demonstrate that exposing neonatal rats to IH, simulating the blood O₂ saturation profiles occurring in recurrent apnea augments the hypoxic sensing by the carotid body and AMC.

1.2.1. The effects of neonatal IH on the carotid body and AMC persist into adult life and their consequences on cardio-respiratory functions

IH exposed rat pups showed augmented hypoxic ventilatory response (Peng *et al*, 2004), a hallmark of carotid body chemosensory reflex, which is similar to the heightened HVR seen in preterm infants (Nock *et al*, 2004). The heightened HVR was associated with higher incidence of apneas in neonatal rat pups (Julien *et al*, 2008). Remarkably, adult rats treated with neonatal IH exhibited: a) augmented carotid body response and CA secretion to hypoxia, b) elevated plasma CA levels, c) high incidence of apneas, and d) hypertension as compared to control rats reared under room air (Nanduri *et al*, 2012). These observations suggest that neonatal IH leads to long-lasting increase in hypoxic sensitivity of the carotid body and AMC and results in serious abnormalities in cardio-respiratory function.

The above findings from neonatal IH rats are reminiscent of recent clinical studies in human subjects. Adults born with apnea of prematurity exhibit higher incidence of sleep-disordered breathing with several co-morbidities including systemic hypertension and metabolic syndrome (Rosen *et al*, 2003; Paavonen *et al*, 2007; Dalziel *et al*, 2007).

1.3. Reactive oxygen species (ROS) - A major cellular mechanism mediating the effects of neonatal IH

Adverse physiological consequences of neonatal IH prompted the examination of the underlying mechanisms. Increased reactive oxygen species (ROS) and the ensuing oxidative stress is an important cellular mechanism mediating cardio-respiratory changes caused by IH in experimental models and in patients with recurrent apneas (Christou *et al*, 2003; Grebe *et al*, 2006; Prabhakar, 2011; Suzuki *et al*, 2006). Previous studies have shown that ROS mediate cellular responses to IH (Prabhakar *et al*, 2007). The following observations demonstrate that ROS mediate the neonatal IH-induced augmented hypoxic sensing in the carotid body and AMC: 1) IH increased ROS levels in neonatal carotid bodies and adrenal medullae as evidenced by elevated malondialdehyde (MDA) levels (Pawar *et al*, 2009; Souvannakitti *et al*, 2010), which represent oxidized lipids and proteins (Hiroshi *et al*, 1979; Peng *et al*, 2003). 2) Rats treated with a stable superoxide dismutase mimetic (MnTMPyP), a potent scavenger of ROS blocked IH-evoked increases in ROS in the carotid body and abolished sensitization of the hypoxic response (Pawar *et al*, 2009); and 3) Anti-oxidants prevented the effects of IH on hypoxia-evoked CA secretion and exaggerated $[Ca^{2+}]_i$ responses in neonatal AMC (Souvannakitti *et al*, 2010).

The elevated ROS levels by IH could be due to either increased generation by pro-oxidant enzymes and/or decreased degradation by anti-oxidant enzymes. The family of NADPH oxidases (NOX) constitutes one of the major sources of ROS generation in mammalian cells

(Bedard & Krause, 2007). NADPH oxidase 2 and 4 (Nox2 and 4) mRNAs as well as the enzyme activities were up regulated in neonatal adrenal medulla. Similar up regulation of Nox2 and 4 was also seen in IH exposed carotid bodies (Peng *et al*, 2009). Anti-oxidant enzymes including the manganese superoxide dismutase or SOD-2, catalase (*CAS1*), and glutathione peroxidase 1 (*GPX2*) are down regulated in carotid bodies and adrenal medullae in adult rats exposed to neonatal IH (Nanduri *et al*, 2012). These observations suggest that both decreased degradation by anti-oxidant enzymes and increased generation by NADPH oxidases contribute to elevated ROS levels by neonatal IH, facilitating the hypoxic sensing by the carotid body and the AMC. These findings further demonstrate that neonatal IH leads to long-lasting changes in genes encoding pro-, and anti-oxidant enzyme leading to persistent oxidative stress in the carotid bodies and adrenal medullae in adult rats.

1.4. Neonatal IH initiates epigenetic programming of redox state by DNA methylation

DNA methylation is a key mechanism mediating the long-term regulation of gene expression (Niculescu *et al*, 2005; Waterland & Jirtle, 2003). DNA methylation involves covalent addition of a methyl group to the fifth carbon of the cytosine to form 5-methyl cytosine (5meC). Methylation occurs predominantly in cytosine located at 5' of guanine, known as CpG dinucleotides (CpG), which occur in clusters in the promoter region of genes often referred as "CpG islands". In general, an inverse relationship exists between the extent of methylation of regulatory CpG island(s) and gene transcription (Gardiner-Garden & Frommer, 1987; Illingworth & Bird, 2009). Thus, DNA hypermethylation leads to repression of gene transcription and hypomethylation causes transcriptional activation.

A recent study by Nanduri *et al* (2012) reported that the persistent oxidative stress in adult rats exposed to neonatal IH is in part due to long-lasting down regulation of genes encoding anti-oxidant enzymes in both the carotid body and adrenal medullae involving DNA hypermethylation. Neonatal IH increases DNA hypermethylation of a single CpG dinucleotide within the *Sod2* gene close to the transcription initiation site and was associated with reduced *Sod2* mRNA, protein and enzyme activity.

1.4.1. Role of DNA methyl transferases in regulating anti-oxidant gene methylation

DNA methylation is catalyzed by the DNA methyltransferases Dnmt1, Dnmt3a, and Dnmt3b. Whereas Dnmt1 is responsible for the maintenance of methylation, Dnmt3a and Dnmt3b are *de novo* methylases (Miranda & Jones, 2007). The expression of DnmTs were analyzed in carotid bodies and adrenal medullae in adult rats exposed to IH during neonatal period (Nanduri *et al.*, 2012). The levels of Dnmt1 and Dnmt3b mRNA and protein were elevated in adult rats exposed to neonatal IH, whereas, Dnmt3a mRNA expression was unchanged.

1.4.2. DNA methyl transferase inhibitors prevent the long-lasting effects of neonatal IH

Recent studies revealed direct interactions of Dnmt3a and Dnmt3b with specific transcription factors as an important mechanism for gene regulation by DNA methylation (Hervouet *et al*, 2009). DnmTs can be inhibited pharmacologically by the nucleoside analog

5-aza-2'-deoxycytidine (decitabine), which irreversibly binds to the Dnmts once incorporated into DNA, resulting in global demethylation after several cell divisions (Bird & Wolffe, 1999).

Treating neonatal rats with decitabine, during IH exposure, prevented hypermethylation of the *Sod2* DNA, oxidative stress, the enhanced hypoxic sensitivity of the carotid body and AMC, and the cardio-respiratory abnormalities in adult rats. The effects of decitabine are unlikely to be due to non-selective actions because: (a) basal hypoxic responses of the carotid body and adrenal medulla were unaffected in decitabine treated rats; (b) decitabine had no effect on responses of the carotid body and adrenal medulla to stimuli other than hypoxia; and (c) in control rats, decitabine had no effect on breathing, and blood pressure, redox status and pro-, and anti-oxidant enzyme expressions.

1.5. Summary and Perspective

The findings described above demonstrate a hitherto uncharacterized role for epigenetic modulation of O₂ sensing via DNA hypermethylation in neonates, pre-disposing them to disrupted cardio-respiratory homeostasis in adulthood. Genes encoding anti-oxidant enzymes other than *Sod2* were also down regulated in rats treated with neonatal IH (Nanduri *et al*, 2012); whether DNA methylation contributes to these changes remains to be investigated.

In addition to DNA methylation, post-translational modifications of histones (methylation, acetylation, phosphorylation, and ubiquitination) can change chromatin structure, altering the access of RNA polymerase and transcription factors to the DNA and thereby modulating gene expression (Chi *et al*, 2010). In particular, the relative activities of histone methyltransferases and histone demethylases (JHDMs) provide a dynamic mechanism of transcriptional regulation (Siddiqi *et al*, 2010). HIF-1 activates the transcription of several JHDM genes including *JMJD1A* (*KDM3A*), *JMJD2B* (*KDM4B*), and *JMJD2C* (*KDM4C*) (Pollard *et al*, 2008) and recent studies suggest that JHDMs are important for increasing or maintaining the expression of specific genes that are known to be up-regulated by HIF-1 (Krieg *et al*, 2010; Xia *et al*, 2009). Whether HIF-1 activated JHDMs contribute to persistent changes in pro-oxidant gene expression like NADPH oxidase in response to neonatal IH requires further investigation.

Clinical studies also show that epigenetic modifications may constitute an important determinant of inflammatory phenotype in OSA. The levels of DNA methylation of 24 inflammatory related genes were studied in children with OSA with and without high levels of C-reactive protein and it was concluded that FOXP3 DNA methylation levels may provide a potential biomarker for end-organ vulnerability (Kim *et al*, 2012). In addition, abnormal eNOS-dependent vascular responses in children with OSA was also reported and this phenotype was associated with epigenetic modifications in the eNOS gene (Kheirandish-Gozal *et al*, 2013).

The current findings of epigenetic modulation of hypoxic sensing may also be of considerable relevance for understanding the impact of long-term IH on autonomic function

in adult life. Clinically, this is of great importance because a subset of obstructive sleep apnea (OSA) patients are resistant to continuous positive airway pressure (CPAP) treatment. It is likely that reprogramming by epigenetic mechanisms render these OSA patients resistant to CPAP therapy. Assessing this possibility might lead to the use of DNA hypomethylating drugs as a novel therapeutic intervention to prevent the CPAP resistant comorbidities in OSA patients.

Acknowledgments

This research was supported by grants from National Institutes of Health, Heart, Lung and Blood Institute PO1-HL-90554 and UH2- HL-123610.

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Figure 1.

Time course of the hypoxic sensory response of carotid bodies *ex vivo* from intermittent hypoxia (IH)-exposed and control rat pups reared under normoxia (n = 6 pups in each group; age P5; medium $PO_2 = 32-34 \pm 2$ Torr). Area between dashed lines indicates duration of hypoxic challenges (*modified from Peng et al., 2004*).

Figure 2.

Effects of intermittent (IH) and sustained hypoxia (SH) on hypoxia-evoked catecholamine (CA) secretion from neonatal adrenal medullary chromaffin cells (AMC). *Top panels:* examples of hypoxia-evoked CA secretion from AMC derived from postnatal day 5 (P5) rats reared under normoxia (control) or IH or SH. Horizontal black bar represents the duration of the hypoxia (PO₂ $\approx$ 30 mmHg). *Bottom panels:* average data on the effects of graded hypoxia on the number of secretory events/min (*left*) and the average CA molecules released/event (*right*). PO₂, partial pressure of oxygen (mmHg) in the superfusing medium. Data shown are means $\pm$ SE. Control=17 cells, IH=20 cells and SH=18 cells (SH) obtained from 3 different litters in each group. *P < 0.01; **P < 0.001; n.s., not significant (P > 0.05) (*modified from Souvannakitti et al., 2009b*).