Published in final edited form as:

Respir Physiol Neurobiol. 2008 December 10; 164(1-2): 272–276. doi:10.1016/j.resp.2008.05.017.

POST-TRANSLATIONAL MODIFICATION OF PROTEINS DURING INTERMITTENT HYPOXIA

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Abstract

Post-translational modification (PTM) is one of the mechanisms by which protein function is regulated by chronic hypoxia. This article presents an overview of recent findings on PTM of proteins induced by chronic intermittent hypoxia (CIH) which is experienced by humans with sleep disordered breathing resulting in autonomic abnormalities. The analysis of PTM of proteins involves electrophoretic separation of tissue or cellular proteins followed by immunolabeling using antibodies specific to native and posttranslationally modified forms. Recent results demonstrate that CIH, depending on the pattern, duration and severity of hypoxia, alters the state of phosphorylation of a subset of proteins associated with transcriptional factor activation, signaling pathways and neurotransmitter synthesis via activation of appropriate enzymatic machinery that catalyzes specific phosphorylation reactions. Investigation pertaining to PTMs associated with CIH is at its infant stage and future application of high throughput proteomics techniques are necessary to unravel other important PTMs associated with various critical metabolic and signaling pathways that are activated by intermittent hypoxia.

Keywords

Post-translational modifications; chronic intermittent hypoxia; proteomics; protein phosphorylation; protein kinases; tyrosine hydroxylase and transcription factors

1. Introduction

Molecular oxygen (O_2) is vital for various cellular processes because of its critical role in ATP production via oxidative phosphorylation. Organisms respond to chronic hypoxia (i.e., decreased availability of O_2) by transcriptional activation of genes resulting in de novo protein synthesis. Recent advances in proteomics highlight the importance of posttranslational modification (PTM) as an important mechanism for the functional regulation of existing proteins under chronic hypoxia (for ref see Kumar and Klein, 2004). People living at sea level experience chronic intermittent hypoxia (CIH) more often than continuous hypoxia under a variety of conditions including sleep disordered breathing manifested as recurrent apneas. CIH associated with recurrent apneas leads to autonomic disturbances resulting in cardio-respiratory morbidities. However, only a limited number of studies have investigated the effects of CIH on PTMs of proteins. The purpose of this review is to highlight recent findings on PTM

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of proteins in tissues and cells in response to CIH. Several excellent reviews on various aspects of PTM (Spickett et al., 2006; Unwin et al., 2006; Gevaert et al., 2007; Kiernan, 2007; Reinders and Sickmann, 2007; Witze et al., 2007) are available and these aspects, therefore, will be discussed only briefly.

2. General aspects of post-translational modification of proteins

2.1. Definition, chemical basis and biological significance of PTM

Covalent modification of one or more amino acid side chains of a given protein is often referred to as PTM. Thus far, nearly 300 PTM reactions have been identified. Examples of well studied PTM reactions associated with reactive side chains of amino acid residues are shown in Table 1. PTM can dramatically alter the biological function of a given protein even in the absence of changes in the protein level or transcription. Further, the occurrence of a given PTM reaction depends on the spatial orientation of specific amino acid residue (s) as well as neighboring amino acids that confer selectivity and reactivity via affecting the electrophilic nature of the critical amino acid residue that undergoes modification. Specific enzymes mediate many of the PTM reactions outlined in Table 1.

Some normal cellular processes are known to be regulated via PTM. For instance, protein phosphorylation has been identified as one of the major control mechanisms that govern most aspects of cell life. About one third of mammalian proteins are shown to contain covalently bound phosphates the steady state level of which is controlled by the activities of protein kinases, protein phosphatases and their regulatory subunits. Another example is the post-translational proteolytic processing involving multiple classes of proteases which contribute to constitutive synthesis of biologically active neuropeptides, which function as neurotransmitters/modulators in the peripheral and central nervous systems. Also, post-translational proteolysis serves to generate active enzymes via conversion of inactive zymogen form to active enzyme form (Ex: conversion of trypsinogen to trypsin). PTM also plays important roles in trafficking of macromolecules to different cellular compartments via posttranslational glycosylation (Ex: membrane transport of receptors). Another well studied PTM involves intra-disulfide bond formation between two cysteine residues which is critical in the formation of quaternary structure and functional expression of enzymatic activity of many proteins.

Some of the enzymes associated with PTMs are mono-oxygenases requiring molecular O₂ for their activity. Examples of this class of enzymes include peptidylglycine α -amidating monooxygenase that catalyzes the C-terminal amidation of peptide transmitters/modulators and prolyl hydroxylases that catalyses the hydroxylation of proline residues in hypoxia-inducible factor-1, a transcriptional activator. Several other enzymes associated with PTMs are regulated by red-ox changes. Consequently, O₂ availability as well as red-ox state of the cellular environment can profoundly impact some of the PTM reactions. Given these regulatory features of enzymes that mediate PTM reactions, hypoxia can alter many reactions associated with PTMs (Kumar and Klein, 2004).

3. Methodological approach for the analysis of PTM

In general, two types of approaches are available to assess quantitative changes in the state of PTM of proteins. They are: i) analysis of PTM at the level of individual protein and ii) analysis of PTM at the whole proteome level. Among the two approaches, thus far, the effect of CIH on PTM was investigated primarily at the level of individual protein. Therefore, in the following section a brief description of this method will be presented.

3.1. Analysis of PTM at individual protein level

This approach requires either monoclonal or polyclonal antibodies specific to either native or posttranslationally modified forms of the protein of interest. The analysis, in general, consists of one dimensional SDS-PAGE separation of the protein of interest from either whole cell lysates or tissue extracts or enriched sub-cellular fractions followed by transfer of the proteins to suitable membrane and identification of PTM using specific antibodies. Also, depending on the suitability of the antibodies, either immunocytochemical (qualitative) or ELISA (quantitative) analysis can be performed to assess the state of PTM of protein.

4. Effect of CIH on post-translational modification of proteins

Several studies examined CIH-induced PTMs in a subset of proteins that are associated with transcriptional activation, signaling pathways, transmitter synthesis and cardio-protection in rodents. Most of these studies investigated CIH-induced phosphorylation of target protein substrates using immunological characterization of PTM at a single protein level. In the following sections, a brief description of results derived from these studies will be presented. Thus far, there are no studies that have examined CIH-induced PTMs using proteomics approach.

A summary of CIH-induced PTMs thus far identified in various models of intermittent hypoxia is presented in Table 2. A notable feature of these studies is that the pattern of intermittent hypoxia used in these investigations varies considerably among different investigators. Examples of variations include the duration of hypoxia and normoxia, the total number of episodes, the severity of hypoxia, and the stimulus (hypoxia versus hypoxia + hypercapnia). The studies included in Table 2 addressed changes in PTM in the carotid body, brainstem, hippocampus, cerebral cortex, adrenal medulla, heart, and arteries of rats exposed to various patterns of CIH. The effects of CIH on PTM in pheochromocytoma 12 (PC12), endothelial and adrenal chromaffin cells were also presented. It is evident from the data presented in Table 2 that CIH facilitates phosphorylation of a subset of cellular proteins and in some instances induces dephosphorylation of proteins. In the following sections, major findings on CIH-induced changes in the phosphorylation state of proteins associated with transcriptional activation, signal transduction pathways, and neurotransmitter synthesis will be presented.

4.1. CIH-induced phosphorylation of transcriptional factors and their regulators

Several studies examined the effects of CIH on the phosphorylation state of several transcriptional factors and their regulators such as cAMP responsive element binding (CREB) protein, I- κ B α , a regulator of nuclear factor- κ B (NF- κ B), and hypoxia-inducible factor-1 (HIF-1) using both rodent and cell culture models.

4.1.1. Phosphorylation of CREB—In a series of studies, Gozal and his group investigated the effect of CIH comprising equal duration of alternating cycles of hypoxia and normoxia (90 s each), 12 h per day for 14 days on CREB phosphorylation in adult rat CA1 hippocampal region (Goldbart et al., 2003b; Gozal et al., 2003; Row et al., 2003). These investigators observed that CIH significantly decreased the level of Ser-133 phosphorylated form of CREB (p-CREB) without altering the total CREB expression (Goldbart et al., 2003b). CIH induced a time-dependent decrease in p-CREB which reached a maximum between 6 h and 3 days, and returned to control level by 14–30 days. Aging further facilitated CIH-evoked CREB dephosphorylation (Gozal et al., 2003). Interestingly, CIH-induced CREB dephosphorylation can be partially blocked by subjecting the rats to spatial pre-training prior to CIH exposure (Row et al., 2003). These studies led to the suggestion that CIH-evoked changes in CREB phosphorylation might be of importance in cognitive changes associated with sleep-disordered breathing.

4.1.2. Phosphorylation of NF- κ B and I- κ B α —CIH has been shown to activate nuclear factor κ B (NF- κ B), transcriptional regulator that plays a major role in responses to inflammatory signaling (Greenberg et al., 2006). NF- κ B dimers are held in the inactive state by a family of inhibitors called I- κ B. Activation of a multi-subunit I- κ B kinase (IKK) complex phosphorylates I- κ B leading to its degradation and the liberation of NF- κ B dimer for active transcription. CIH, in a pattern-dependent manner, differentially altered the phosphorylation state of I- κ B α in rat brain. For instance, exposure of rats to 12 h of 10% O₂ per day for 8 days resulted in the reduction of phosphorylated form of I- κ B α in the cortex (Rapino et al., 2005; Bianchi et al., 2006). On the other hand, exposure of endothelial cells to short-duration of intermittent hypoxia (5 min) and reoxygenation (10 min) up to 16 cycles increased I- κ B α phosphorylation and its subsequent degradation (Ryan et al., 2007).

4.1.3. Phosphorylation of HIF-1 α —HIF-1 is a transcriptional activator that regulates the expression of multiple genes during continuous hypoxia (Semenza, 2004). HIF-1 is composed of a constitutively expressed HIF-1 β and O₂ regulated HIF-1 α subunit. A recent study examined the effects of CIH on phosphorylation of the HIF-1 α subunit in endothelial cells (Toffoli et al., 2007). Exposure of endothelial cells to four repeated cycles of 1h hypoxia followed by 30 min normoxia increased the phosphorylated form of HIF-1 α in a cycle-dependent manner with a greater increase observed at the end of 4th cycle than that seen in previous cycles. The increase in HIF-1 α phosphorylation which is mediated via a PKA-dependent mechanism has been implicated in increased HIF-1 transcriptional activity and contributes to enhanced cell survival under these experimental conditions (Toffoli et al., 2007).

4.2. CIH and protein kinases

4.2.1. Protein kinase C (PKC)—Multiple isoforms of protein kinase C (PKC) are expressed in a variety of cells. Some of the PKC isoforms are activated by Ca²⁺ and others by diacylglycerol. PKC-dependent phosphorylation plays important roles in a variety of cellular processes. Exposure of mice to CIH (15 s of hypoxia followed by 5 min of normoxia, 9 episodes per h, 8 h per day) for 4 days activates Ca²⁺ sensitive PKC isoform(s) in the adrenal medulla which contributes to increases in readily releasable pool of catecholamine vesicles (Kuri et al., 2007). Rats exposed to 14 days of CIH (1 min hypoxia/hypercapnia followed by 2 min normoxia) exhibit endothelin 1 (ET-1)-dependent hypertension and increased vascular resistance which was attributed to increased activation of PKC- δ by ET-1 (Allahdadi et al., 2008). Yuan et al (2005, 2008) reported activation of Ca²⁺ sensitive PKC α and γ isoforms in PC12 cells which contributes to IH-induced HIF-1 mediated transcription via activation of mammalian target of rapamycin (mTOR).

4.2.2. Protein kinase B (Akt)—Akt (protein kinase B) is an oncogene that is involved in cellular survival pathways via inhibition of apoptotic pathways. In rats exposed to 30 days of CIH (90 s hypoxia followed by 90 s normoxia), Goldbart et al (2003a) reported a biphasic alteration in Akt phosphorylation; an initial decrease reaching a maximum between 6h to 3 days followed by return to baseline levels by 30 days. The alterations in Akt phosphorylation were attributed to CIH-induced initial increases and subsequent decreases in neuronal apoptosis. On the other hand, acute intermittent hypoxia (AIH; 3 $\times$ 90 s hypoxia at 10 min intervals) significantly increased Akt phosphorylation in the myocardium of rats fed ad libitum compared to unfed rats (Wu et al., 2007). Because Akt phosphorylation was implicated in the regulation of glycogen synthase, it was suggested that AIH-evoked increase in Akt phosphorylation may play a role in glycogen utilization.

4.2.3. Differences in the activation pattern of mitogen activated protein kinases (MAPKs) and Ca²⁺-calmodulin-dependent (CaM) kinase by continuous and

intermittent hypoxia—Intermittent hypoxia activates mitogen-activated protein kinases (MAPK) (ERK1 and 2; Jun kinase), and CaMK II in PC12 cells (Yuan et al., 2005). Comparison of PC12 cell responses to continuous hypoxia revealed notable differences in the above outlined protein kinase activations by IH. For instance, IH resulted in a sustained activation (5.5 fold increase; Yuan et al., 2005), whereas continuous hypoxia resulted in a transient (15 min) and modest (1.5 fold increase) activation of CaM kinase (Premkumar et al., 2000). Likewise, Jun kinase was activated only by IH but not by continuous hypoxia, whereas continuous but not IH activated p38 kinase in PC 12 cells (Yuan et al., 2005; Premkumar et al., 2000). Functional analysis revealed that ERK-mediated signaling is critical for HIF-1 activation by continuous hypoxia (Conrad et al., 1999; Seta et al., 2003) but not by IH (Yuan et al., 2005). Thus, although both IH and continuous hypoxia activates similar protein kinases, the kinetics of activation and the downstream targets seem to differ between the two forms of hypoxia (Nanduri and Prabhakar, 2007).

Acute IH (AIH; 40 s 10% O₂ and 20 s 21% O₂ for 4h) increases Erk1/2 as well as p38 phosphorylations in rat myocardium (Beguín et al., 2007). Inhibitors specific to p38MAPK and Erk1/2 prevented AIH-induced cardio-protection against ischemic insult. Chen et al (2007) found cardiac hypertrophy in rats exposed to long term intermittent hypoxia (8 h 12% O₂ per day for 4 or 8 weeks) and this effect was attributed to increased p38MAPK activation.

4.3. Transmitter synthesis

4.3.1. Tyrosine hydroxylase (TH)—Humans with recurrent apneas and rats exposed to CIH had elevated levels of circulating catecholamines (Fletcher et al., 1987; Phillips and Somers, 2000). These observations suggest that CIH facilitates catecholamine release from both adrenal medullary and non-adrenal medullary sources. Using an in vitro adrenal medullary preparation, Kumar et al (2006) showed that CIH not only increased catecholamine content in the adrenal medulla but also facilitated hypoxia-evoked catecholamine efflux which was absent in the control adrenal medulla. These observations indicate that CIH might impact on catecholamine synthesis.

Catecholamine synthesis in vivo is regulated by the activity of the rate-limiting enzyme, tyrosine hydroxylase (TH) which is under the control of serine phosphorylation at multiple serine residues including Ser-19, Ser-31 and Ser-40 (Dunkley et al., 2004; Lehmann et al., 2006; Bobrovskaya et al., 2007). Four studies independently examined the effects of different patterns of intermittent hypoxia on TH activity using both cell culture and rodent models. Using a PC12 cell culture model, Kumar et al (2003) investigated the effect of short-duration intermittent hypoxia (SDIH) comprising 15 s of 1% O₂ and 3 min of 21% O₂ per cycle for 60 cycles on TH activity. Their results showed that SDIH increased TH activity by elevating the level of Ser-40 phosphorylated form of TH without altering the total TH protein expression. More importantly, these authors showed that pre-treatment with CaM kinase and PKA-specific inhibitors attenuated SDIH-induced increases in enzyme activity and Ser-40 phosphorylation of TH. These results collectively suggest that SDIH activates TH in PC12 cells via Ser-40 specific phosphorylation mediated in part by CaMK and PKA. Rats exposed to a similar paradigm of intermittent hypoxia for 10 days showed a marked increase in TH activity and serine phosphorylation of TH, and elevation in catecholamine levels in brainstem regions. However, a similar such increase in either TH activity or serine phosphorylation of TH was not seen in brain regions of rats exposed to long-duration intermittent hypoxia comprising 4 h of hypobaric hypoxia per day for 10 days (Kumar et al., unpublished observations).

Using a different paradigm of CIH (90 s hypoxia and 90 s normoxia, 12 h per day for up to 7 to 30 days), Gozal et al (2005) reported decreases in serine phosphorylation of TH in the brainstem, but modest increases in cerebral cortex. With a similar CIH paradigm, Hui et al (2003) found only a modest increase in serine phosphorylation of TH in the carotid body in

contrast to robust increases in TH phosphorylation by continuous hypoxia. Further, the effects of CIH and continuous hypoxia on serine phosphorylation of TH appeared to be specific to the carotid body, because such changes were not seen either in the adrenal medulla or superior cervical ganglion. Thus, taken together, these studies indicate that TH-phosphorylation and hence catecholamine synthesis critically depends on the CIH paradigm.

5. Summary and Future Directions

The above results provide an over view of our current understanding on the impact of various forms of chronic intermittent hypoxia on phosphorylation of a subset of cellular proteins. Since more than 300 PTMs have been documented, it will be of considerable interest to determine whether other types of posttranslational events are also activated in response to intermittent hypoxia. Thus far, all the available information on CIH-induced changes in phosphorylation is exclusively derived from immunochemical analysis of individual protein of interest. To understand the full impact of CIH on protein phosphorylation and also on other PTMs in various cellular compartments altering cellular functions, “phospho-proteomics” approach (Spickett et al., 2006) needs to be applied. Given the fact that CIH associated with sleep disordered breathing is one of the major contributing factors in a variety of autonomic abnormalities resulting in hypertension, metabolic syndrome and stroke, unraveling dynamic changes in PTMs during the progression of CIH-induced abnormalities will enable in the identification of novel therapeutic targets.

Acknowledgements

The authors thank Dr. Vandana Rai for her scientific contribution to studies related to tyrosine hydroxylase reported in this review. This study was supported by National Institutes of Health Grants PO1HL-90554 (to N. R. P) and RO1HL-89616 (to G. K. K).

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Table 1

Examples of post-translational modification reactions and their target amino acid residues in proteins

No.	Amino acid residues (functional reactive group)	Types of Modification (enzymatic or non-enzymatic)
1	Serine/threonine (-OH)	Phosphorylation (enzymatic)
2	Cysteine (-SH)	Thiolation (enzymatic), methylation (enzymatic) and oxidative modification (non-enzymatic)
3	Lysine (-ε-NH ₂)	Acetylation (enzymatic), biotinylation (enzymatic), hydroxylation (enzymatic)
4	N-terminal of proteins (-α-NH ₂)	Phrenylation, pyroglutamylation (enzymatic)
5	Glutamine (-CONH ₂)	Deamination (non-enzymatic)
6	Tyrosine (-OH)	Nitration, sulfation (non-enzymatic), phosphorylation (enzymatic)
7	Methionine (-SCH ₃)	Sulfenylation via oxidative modification
8	Tryptophan (indole ring)	Oxidative modification
9	Proline	Hydroxylation (enzymatic)
10	Asparagine	Glycosylation (enzymatic), hydroxylation (enzymatic)
11	Peptide-Lys/Arg-Lys/Arg-peptide	Proteolytic cleavage (enzymatic)
12	Peptide-Gly	C-terminal α-amidation (enzymatic)

Table 2
Intermittent hypoxia and posttranslational protein phosphorylation

No	Tissue/Cell	CIH pattern	Target Proteins	Type and response of PTM	Ref
1.	Aged rat cerebral cortex	12 h at 10% O ₂ + 12 h at 21% O ₂ for 8 days	I-κBα	Decreased phosphorylation	Rapino et al., 2005
2	Adult rat CA1 hippocampal region	90 s at 10% O ₂ + 90 s at 21% O ₂ , per 12 h light cycle, for 14 days	CREB	Decreased phosphorylation (Ser-31)	Goldbart et al., 2003b; Gozal et al., 2003
		+ pretraining	CREB	Attenuation of CIH-induced decrease in CREB phosphorylation	Row et al., 2003
3.	Adult rat CA1 hippocampal region	90 s at 10% O ₂ + 90 s at 21% O ₂ , per 12 h light cycle, for 14 days	Protein kinase B (Akt)	Decreased phosphorylation	Goldbart et al., 2003a
4.	Adult rat (fed) heart	3, 90 s apnea at 10 min intervals	Akt	Increased phosphorylation	Wu et al., 2007
5.	Adult rat heart	Intermittent high altitude hypoxia (6 h at 0.4 atm for 42 days)	Phospholam ban	Increased phosphorylation (Ser-16 via PKA)	Xie et al., 2005
6.	Adult rat heart	40 s at 10% O ₂ + 20 s at 21% O ₂ for 4 h	p38MAPK and Erk1/2	Increased phosphorylation after 24 h	Beguin et al., 2007
7.	Adult rat left ventricle	8h at 12% O ₂ per day for 4 weeks	p38MAPK	Increased phosphorylation	Chen et al., 2007
8.	Young and aged rat myocardium	12 h at 10% O ₂ + 12 h at 21% O ₂ for 8 days	p66	Increased Tyr-phosphorylation	Bianchi et al., 2006
9.	Rat small mesenteric artery	Eucapnic intermittent hypoxia (1 min at 5% O ₂ / 5% CO ₂ , 2 min at 21% O ₂ / 0% CO ₂ , 20 cycles per h, 7 h per day for 14 days	PKC-δ	Increased response to ET-1	Allahdadi et al., 2008
10.	Adult rat cerebral cortex and brainstem	90 s at 10% O ₂ + 90 s at 21% O ₂ , per 12 h light cycle, for 7 days	TH	Increased phosphorylation (Ser-40)	Gozal et al., 2005
11.	Adult rat carotid body	90 s at 10% O ₂ + 90 s at 21% O ₂ , per 12 h light cycle, for 1–30 days	TH	Increased phosphorylation (Ser-19, Ser-31 and Ser-40)	Hui et al., 2003
12.	PC12 cells	15 s at 1% O ₂ + 3 min at 21% O ₂ for 60 cycles	TH	Increased phosphorylation (Ser-40 via PKA and CaMK)	Kumar et al., 2003
13.	Adult mouse adrenal chromaffin cells	15 s at 5% O ₂ + 5 min at 21% O ₂ ; 8h per day for 4 days	PKC	Increased phosphorylation (Thr-514)	Kuri et al., 2007

No	Tissue/Cell	CIH pattern	Target Proteins	Type and response of PTM	Ref
14.	Endothelial cells	Intermittent hypoxia / reoxygenation	I- κ B α	Increased phosphorylation	Ryan et al., 2007
15.	Endothelial cells	60 min at hypoxia + 30 min at normoxia for 4 cycles	HIF-1 α	Increased phosphorylation (via PKA)	Toffoli et al., 2007
16.	PC12 cells	30 s at 1.5%O ₂ + 5 min at 20%O ₂ for 60 cycles	HIF-1 α	PKC activation	Yuan et al., 2005, 2008