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TRANSCRIPTIONAL RESPONSES TO INTERMITTENT HYPOXIA

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Abstract

Recurrent apneas are characterized by transient repetitive cessations of breathing (two breaths duration or longer) resulting in periodic decreases in arterial blood PO₂ or chronic intermittent hypoxia (IH). Patients with recurrent apneas and experimental animals exposed to chronic IH exhibit cardio-respiratory morbidities. The purpose of this article is to highlight the current information on the transcriptional mechanisms associated with chronic IH. Studies on rodents and cell cultures have shown that IH activates a variety of transcription factors including the hypoxia-inducible factor-1 (HIF-1), *c-fos* (immediate early gene), nuclear factor of activated T-Cells (NFAT), and nuclear factor kB (NF-kB). The signaling pathways associated with transcriptional activation associated with IH differ from continuous hypoxia (CH). Compared to same duration and intensity of CH, IH is more potent in activating HIF-1 and *c-fos* and also results in long-lasting accumulation of HIF-1 α and *c-fos* mRNA, a phenomenon that was not seen with CH. IH-evoked transcriptional activation by HIF-1, *c-fos* as well as the resulting activator protein-1 (AP-1) requires reactive oxygen species (ROS)-mediated signaling and involves complex feed-forward interactions between HIF-1 and ROS. Chronic IH evoked cardio-respiratory responses are absent in *Hif-1a*^{+/-} mice, and hypertension elicited by chronic IH is absent in mice lacking NFAT3c. These studies indicate that cardio-respiratory responses to chronic IH depend on complex interactions between various transcription factors resulting in alterations in several downstream genes and their protein products.

Keywords

Hypoxia-inducible factor-1; NFAT; Activator protein-1; Nuclear factor kB; intermittent hypoxia; reactive oxygen species; NADPH oxidase

1. Introduction

Recurrent apneas are characterized by transient repetitive cessations of breathing (two breaths duration or longer) resulting in cyclical decreases in arterial blood PO₂ or chronic intermittent hypoxia (IH). An estimated 4–5% of adult males, 2–4% of females after

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menopause, and 50–70% of premature infants experience chronic IH as a consequence of recurrent apneas (Nieto et al., 2000; Poets et al., 1994). Patients with recurrent apneas exhibit cardio-respiratory co-morbidities including pulmonary as well as systemic hypertension, myocardial infarction, stroke, ventilatory abnormalities, and sudden death (Shahar et al., 2001). Similar cardio-respiratory changes were also reported in rodents exposed to chronic IH (reviewed in Prabhakar et al., 2007). Studies on rodents and cell cultures have shown that IH activates several transcription factors. The purpose of this article is to summarize what is currently known on the effects chronic IH on activation of transcriptional factors, underlying mechanisms and the potential contribution of transcriptional activators on chronic IH- evoked cardio-respiratory responses. In contrast to IH associated with recurrent apneas, wherein each hypoxic episode lasts no more than couple of breaths, exposing individuals to few hours of hypoxia per day for a few weeks, which is also intermittent in nature, improves cardio-respiratory functions (Serebrovskaya et al., 1999). Due to constraints of space, this article focuses on transcriptional responses to IH simulating recurrent apneas only.

2. Hypoxia and transcription factors

Hypoxia activates several genes via recruiting specific transcription factors. The resulting protein products maintain homeostasis by enhancing tissue perfusion, ATP generation, glycolysis etc. Transcriptional activators that are affected by continuous hypoxia (CH) include: hypoxia inducible factors (HIF-1 and HIF-2); nuclear factor kappa B (NF- κ B), cyclic AMP response element binding protein (CREB), activating protein-1 (AP-1), p53, early growth response-1 (Egr-1), nuclear factor for interleukin 6 (NF-IL6) (Cummins and Taylor, 2005). With the exception of HIF-1, the effects of hypoxia on other transcription factors can be cell-type and cell-state specific. The following section summarizes the effects of IH on some of these transcription factors.

3. IH and Hypoxia Inducible Factor-1 (HIF-1)

HIF-1 is a heterodimeric protein that is composed of a constitutively expressed HIF-1 β subunit and an O₂-regulated HIF-1 α subunit (Wang et al., 1995). HIF-1 mediated transcriptional activation requires increased HIF-1 α expression, dimerization with HIF-1 β and interaction with co-activators p300 (adenovirus E1A-associated 300-kDa protein) and CBP (cyclic AMP-responsive element-binding protein).

3.1. IH and HIF-1 α protein expression

IH up-regulates HIF-1 α protein in the central nervous system of mice (Peng et al 2006), and in PC12 cell cultures (Yuan et al., 2005). Lam et al. 2008 reported an increase in the HIF-1 α transcript but not the protein during IH. HIF-1 α accumulation by CH requires decreased O₂-dependent proline hydroxylation, ubiquitination, and proteasomal degradation of the HIF-1 α subunit (Coleman and Ratcliffe, 2007). The mechanism associated with IH-evoked HIF-1 α accumulation is complex and requires not only decreased proline hydroxylation but also increased protein synthesis via activation of mTOR (mammalian target of rapamycin) as summarized in Figure 1. Recent study by Yuan et al (2008) reported that ROS generated by NADPH oxidase and the resulting changes in intracellular Ca²⁺ are the primary signaling events that trigger HIF-1 α accumulation by IH.

The effects of IH and CH on HIF-1 α accumulation differ in the following aspects: a) for a given intensity and duration, IH is more potent in increasing HIF-1 α protein than CH (Yuan et al. 2005), and b) following IH, HIF-1 α levels remain elevated during re-oxygenation, whereas they return to control levels within 10 min of re-oxygenation following CH (Yuan et al., 2008). The persistent accumulation of HIF-1 α protein during re-oxygenation

following IH requires increased protein synthesis via activation of mTOR signaling (Yuan et al., 2008).

3.2. IH and HIF-1 mediated transcriptional activation

The effects of IH on HIF-1 mediated transcriptional activation were examined in PC12 cells (Yuan et al., 2005). IH activated HIF-1-dependent transcriptional activity in a stimulus-dependent manner. Like HIF-1 α protein expression, CH of comparable, cumulative duration of IH was ineffective in activating HIF-1-dependent transcription. Previous studies showed that mitogen-activated protein kinases (MAPKs) and phospho-inositol-3 (PI-3) kinases are critical for continuous hypoxia evoked activation of HIF-1 mediated transcription (Sang et al., 2003; Seta et al., 2003). Although MAPKs (ERK-1 & 2; Jun Kinase) are activated by IH, inhibitors of MAPKs and PI-3 kinase were ineffective in blocking IH-elicited activation of HIF-1 mediated transcriptional activation (Yuan et al., 2005).

HIF-1 activation by IH was inhibited by BAPTA-AM, an intracellular Ca²⁺ chelator (Yuan et al., 2005), suggesting the involvement of Ca²⁺ signaling pathways. Calcium-calmodulin-dependent kinases (CaM kinases) are one of the important Ca²⁺ signaling molecules. PC12 cells express CaMK-II and CH causes a transient and modest increase in CaM kinase activity (Premkumar et al., 2000). In striking contrast, IH resulted in robust and persistent activation of CaM kinase (~ 5-fold activation) and more importantly, CaM kinase inhibitor KN93 prevented HIF-1 transcriptional activation, but not HIF-1 α accumulation by IH (Yuan et al., 2005).

Transcriptional activation by HIF-1 requires N- and C- terminal transactivation domains (N-TAD and C-TAD), which are separated by intervening inhibitory domain. FIH-1 (factor inhibiting HIF-1) binds to the inhibitory domain (Mahon et al., 2001) and mediates the O₂-dependent hydroxylation of asparagine (Asn-803), which prevents binding of the co-activators. CaMK II stimulates C-TAD domain function of HIF-1 via a mechanism that is independent of asparaginyl hydroxylation (Yuan et al., 2005). Several lines of evidence suggest that phosphoproteins p300 and CBP (Yaciuk and Moran, 1991) are the major co-activators for HIF-1 activation (Sang et al., 2003; Ruas et al., 2002; Dames et al., 2002). Hypoxia leads to hyperphosphorylation of p300 in PC12 cells via Ca²⁺ signaling by IP-3 receptors (Zakrzewska et al., 2005). CaMK II phosphorylated p300 *in vitro* (Yuan et al., 2005) and CaM kinase inhibitor, KN-93 prevented activation of p300 by IH. These observations suggest that IH induced HIF-1 transcriptional activity is mediated by a novel signaling pathway involving phosphorylation of p300 by CaM kinase (Figure 1).

3.3. Physiological significance of IH-induced HIF-1 activation

3.3.1. Cardio-respiratory responses to chronic IH—Chronic IH has profound effects on cardio-respiratory physiology. Rodents exposed to chronic IH exhibit elevated blood pressures (Fletcher, 2001; Kumar et al., 2006; Peng et al., 2006; Kanagy et al., 2001), increased plasma catecholamine (Bao et al., 1997; Kumar et al., 2006; Peng et al., 2006), and endothelin (a peptide vasoconstrictor) levels (Kanagy et al., 2001). Basal sympathetic nerve activity was elevated in chronic IH exposed rats (Sica et al., 2000) and in recurrent apnea patients (Somers et al., 1995). Furthermore, acute hypoxia as well as hypoxic-hypercapnia-evoked sympathetic excitation was more pronounced in chronic IH-exposed rats (Sica et al., 2000).

Ventilatory response to acute hypoxia is biphasic with an initial augmentation of breathing followed by a decline. The excitatory phase of the hypoxic ventilatory response (HVR) was augmented in chronic IH exposed cats (Rey et al., 2004), rats (Peng et al., 2003; Reeves and Gozal, 2006) and mice (Peng et al 2006). In addition, chronic IH also affects the ventilatory

decline phase of the HVR (Reeves and Gozal, 2006) and augments ventilatory response to hypercapnia (Peng et al., 2006). Repetitive hypoxia leads to long-lasting activation of breathing, a phenomenon termed as "long-term facilitation" (LTF; Mitchell and Johnson, 2003). Prior conditioning with chronic IH augments LTF of breathing (McGuire et al., 2003; Peng et al., 2003; Reeves and Gozal, 2006).

Much of the cardio-respiratory changes for a given stimulus are reflex in nature and are regulated by sensory information from sensory receptors as well as processing of afferent inputs at the central nervous system. Carotid bodies are the primary sensory organs for detecting changes in arterial blood oxygen. It has been proposed that carotid bodies constitute the "frontline" defense system for detecting systemic hypoxia associated with apneas (Cistulli and Sullivan, 1994; Kara et al., 2003). Recent studies have shown that chronic IH leads to enhanced sensory response to acute hypoxia (Peng and Prabhakar, 2003, 2004; Rey et al. 2004; Peng et al., 2006) and long-lasting activation of baseline discharge, a phenomenon termed as sensory LTF (Peng et al., 2003, 2006). Stimulation of carotid body leads to increases in blood pressure, sympathetic excitation and breathing. Consequently, it was proposed that chronic IH-evoked sensory LTF of the carotid bodies contributes to persistent sympathetic activation and hypertension, whereas sensitization of the hypoxic sensory response may lead to instability of the respiratory control system, perpetuating apneas (Prabhakar et al., 2007).

The following section summarizes the potential contribution of HIF-1 activation to the above described alterations in cardio-respiratory responses and carotid body function elicited by chronic IH.

3.3.2. Heterozygous deficiency of HIF-1 α impairs cardio-respiratory and carotid body responses to chronic IH—Complete HIF-1 α deficiency results in embryonic lethality at mid-gestation, whereas *Hif1 α* ^{+/-} heterozygous (HET) mice, which are partially deficient in HIF-1 α expression, develop normally and are indistinguishable from wild type (WT) littermates under normoxic conditions (Iyer et al., 1998; Yu et al., 1999). Wild type mice exposed to chronic IH exhibited: augmented hypoxic ventilatory response; LTF of breathing; enhanced carotid body response to graded hypoxia and sensory LTF; increased blood pressures; and elevated plasma norepinephrine levels. In striking contrast, in HET mice exposed to chronic IH, carotid body responses to hypoxia were absent and all measured cardio-respiratory responses were either absent or markedly attenuated (Peng et al., 2006). HIF-1 α protein expression increased in WT mice, but not in HET littermates exposed to chronic IH. Thus, the virtually complete absence of ventilatory and cardiovascular responses to chronic IH in HET mice could be attributed to lack of induction of HIF-1 α protein expression in these mice.

What are the HIF-1 regulated genes that might contribute to chronic IH-evoked cardio-respiratory changes? Earlier studies have shown that hypoxia-evoked up-regulation of pre-pro ET-1 requires HIF-1 (Hu et al., 1998, Yamashita et al., 2001). Recent studies showed that ET-1 contributes to chronic IH-evoked increases in blood pressure (Kanagy et al., 2001) as well as to the sensitization of the carotid body response to acute hypoxia (Rey et al., 2004). It is likely that HIF-1 contributes to chronic IH-induced changes in blood pressure and carotid body in part by up-regulating the gene encoding pre-pro ET-1. While this remains an attractive notion, further studies are needed to test this possibility and identify other HIF-1 regulated genes that may contribute to cardio-respiratory changes elicited by chronic IH.

3.3.3. Role of reactive oxygen species (ROS) in cardio-respiratory responses to chronic IH and evidence for feed forward interactions of HIF-1 with ROS—It

was proposed that reactive oxygen species (ROS) are generated during the re-oxygenation phase of IH and ROS contribute to chronic IH-evoked physiological responses (Prabhakar, 2001). Indeed, chronic IH increases ROS in several tissues including the carotid body (Peng et al., 2003), adrenal medulla (Kumar et al., 2006), and brainstem (Ramanathan et al., 2005; Row et al., 2003; Veasy et al., 2004). The chronic IH-induced increase in ROS appears to arise in part from the inhibition of the mitochondrial electron transport chain (ETC) specifically at complex I (Yuan et al., 2004) as well as from activation of NADPH oxidase (Zhan et al., 2005; Yuan et al., 2008). More importantly, treating chronic IH exposed rodents with anti-oxidants MnTMPyP [manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride] or NAC [N-acetyl cysteine] prevent chronic IH-evoked cardio-respiratory responses (Peng et al., 2003, Peng and Prabhakar, 2004; Kumar et al., 2006; Troncoso Brindeiro et al., 2007) and changes in carotid body function (Peng et al., 2003 a and b). Anti-oxidants also prevent HIF-1 activation by IH in mice (Peng et al., 2006) and cell cultures (Yuan et al., 2008). Intriguingly, chronic IH elevates ROS levels in WT but not in HET mice deficient in HIF-1 α (Peng et al., 2006). These observations indicate complex positive interactions between HIF-1 and oxidants. It is likely that chronic IH may initially trigger an increase in ROS levels either by inhibiting mitochondrial electron transport chain at complex I or by activating NADPH oxidase. The increased ROS in turn up-regulates HIF-1 α . Once HIF-1 is activated, it may promote persistent increase in ROS either by stimulating oxidants or by inhibiting antioxidants (Figure 2), a possibility that require further studies.

4. IH and Nuclear Factor of activated T-Cells (NFAT)

The role of NFAT family of transcription factors which include NFATc 1, 2, 3 and 4, and NFAT5/TonEBP (Rao et al., 1997), in genetic regulation of immune response is extensively studied. However, recent studies have shown that NFAT transcription factors also play a role in variety of other physiological systems including the nervous (Nguyen and Di Giovanni, 2008) and vascular systems (Wada et al., 2002, Amberg et al., 2004). A rise in intracellular Ca²⁺ and subsequent activation of calcineurin is necessary for NFAT activation (Jain et al., 1995). Calcineurin dephosphorylates several residues in the translocation domain of NFAT resulting in nuclear import. NFAT activation, although increases expression of certain genes (Hogan et al., 2003), it may also repress expression of other genes, especially those encoding K⁺ channels (Amberg et al., 2004; Nieves-Cintrón et al., 2006). A recent study by de Frutos et al (2008) examined the effects of chronic IH on NFATc3 transcriptional activity and its significance in blood pressure changes by IH. These investigators reported increased NFATc3 transcriptional activity in aorta and mesenteric arteries and elevated blood pressures in mice exposed to chronic IH, and these responses were either absent or attenuated in NFATc3 deficient mice or by treating wild type mice with cyclosporine, an inhibitor of calcineurin. ET-1 is a potent activator of NFAT (Stevenson et al., 2001). Because chronic IH elevates ET-1 in rodents, de Frutos et al (2008) suggested that IH-induced increases in blood pressure may involve ET-1 mediated NFAT activation. Since ET-1 is a HIF-1 regulated down-stream gene, it is likely that IH-induced activation of NFATc3 involves HIF-1 mediated up-regulation of pre-pro-ET-1. In addition, NFAT is notable for its ability to bind cooperatively with other transcription factors such as the activator protein-1 (AP-1; Fos/Jun; *see below*) to regulate gene transcription.

5. IH and Activator Protein-1 (AP-1)

The protein products encoded by immediate early genes (e.g., *c-fos*, *c-jun*) form either homo (Jun/Jun) - or heterodimeric (Fos/Jun) protein complexes designated activator protein-1 (AP-1) that drives transcription of a variety of genes (Morgan and Curran., 1989).

5.1. IH activates *c-fos* transcription

Greenberg et al. (1999) reported increased levels of c-Fos protein in the central nervous system of chronic IH exposed rats. IH also increases *c-fos* mRNA expression in cell cultures, which was in part due to increased transcriptional activation (Yuan et al., 2004). IH-evoked *c-fos* transcription requires Serum Response Element (SRE), Calcium Response Element (CRE) but not the AP-1/CRE like *cis*-element (FAP). Interestingly, the magnitude of *c-fos* activation by IH was dependent on the duration of re-oxygenation between the hypoxic episodes rather than the duration of the hypoxic episodes itself. Exposure to comparable cumulative duration of CH had virtually no effect on *c-fos* mRNA expression and *c-fos* promoter activation (Yuan et al., 2004). Following IH, *c-fos* mRNA remained elevated for at least 3 h during the re-oxygenation, whereas it returned to control levels within 1h of re-oxygenation following CH (Yuan et al., 2004). The mechanisms by which IH leads to long lasting increase in *c-fos* mRNA, however, remain to be studied.

5.2. *c-fos* is required for AP-1 transcriptional activation by IH

IH increases AP-1 transcriptional activity in PC12 cells and antisense *c-fos* abolished this effect (Yuan et al., 2004), suggesting that heterodimerization of Fos/Jun is required for AP-1 activation. Anti-sense *c-fos* also blocked IH-induced tyrosine hydroxylase (TH) mRNA expression, an AP-1 regulated gene (Yuan et al., 2004). Since TH is the rate limiting enzyme in catecholamine biosynthesis, the elevated catecholamine levels reported in IH exposed rodents (Bao et al., 1997, Kumar et al., 2006; Peng et al., 2006) and recurrent apnea patients (Ziegler et al., 1997) might in part be due to AP-1 mediated up-regulation of the *TH* gene.

6. IH and Nuclear Factor κ B (NF- κ B)

NF- κ B is an important transcriptional regulator of inflammatory mediators. Ryan et al (2006) reported increased NF- κ B activity in monocytes derived from obstructive sleep apnea patients and this effect was associated with elevated serum tumor necrosis factor- α (TNF- α) and interleukin-8 (IL-8) levels (downstream gene products of NF- κ B). Elevated TNF- α levels correlated with arterial O₂ de-saturation, and treatment of apneas with continuous positive airway pressure (CPAP) normalized TNF- α levels. Similar increase in NF- κ B activation was also reported in mice exposed to IH as well as white blood cells of apnea patients (Greenberg et al., 2006). IH also stimulates NF- κ B-mediated transcriptional activation in HeLa cells (Ryan et al., 2005). However, the mechanisms by which IH activates NF- κ B remain to be investigated.

6.1. IH and possible interactions between NF- κ B and HIF-1

Recent studies suggest interactions between NF- κ B and HIF-1. Belaiba et al (2007) reported that NF- κ B plays a role in HIF-1 α mRNA induction by hypoxia while Rius et al (2008) showed that NF- κ B is critical for hypoxia-evoked HIF-1 α accumulation as well as HIF-1 mediated transcription in the liver and brain. HIF-1 α promoter contains active NF- κ B binding sites at -197/188 upstream of the transcription start site (van Uden et al., 2008). Since IH activates both HIF-1 and NF- κ B it is of interest to examine the mechanisms involved in the interaction of these two transcriptional activators.

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Figure 1. NADPH oxidase signaling in intermittent hypoxia-induced HIF-1 α protein expression and HIF-1 transcription

Key: CIH, chronic intermittent hypoxia; ROS, reactive oxygen species; PLC γ , phospholipase C gamma; IP-3, inositol triphosphate; PKC, protein kinase C; mTOR, mammalian target of rapamycin; HIF-1, hypoxia-inducible factor-1

Figure 2.

ROS-signaling in chronic intermittent hypoxia-evoked cardio-respiratory responses

Key: CIH, chronic intermittent hypoxia; ROS, reactive oxygen species; HIF-1, hypoxia-inducible factor-1; ET-1, endothelin-1; “+” represents positive feed-forward mechanism