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ROS Signaling in Systemic and Cellular Responses to Chronic Intermittent Hypoxia

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ABSTRACT

Chronic intermittent hypoxia (CIH) is a common and life-threatening condition that occurs in many different diseases, including sleep-disordered breathing manifested as recurrent apneas. Reactive oxygen species (ROS) have been identified as one of the causative factors in a variety of morbidities. The purpose of this article is to present a brief overview of recent studies implicating a critical role of ROS in evoking phenotypic adverse effects in experimental models of CIH and in patients with recurrent apneas. In experimental models, CIH activates ROS signaling that contributes to several systemic and cellular responses that include (a) altered carotid body function, the primary chemoreceptor for sensing changes in arterial blood O_2; (b) elevated blood pressures; (c) enhanced release of transmitters and neurotrophic factors; (d) altered sleep and cognitive behaviors; and (e) activation of second-messenger pathways and transcriptional factors. Considerable evidence indicates elevated ROS levels in patients experiencing CIH as a consequence of recurrent apneas. Antioxidants not only prevent many of the CIH-evoked physiologic and cellular responses in experimental settings, but more important, they also offer protection against certain phenotypic adverse effects in patients with recurrent apneas, suggesting their potential therapeutic value in alleviating certain morbidities associated with recurrent apneas. *Antioxid. Redox Signal.* 9, 1397–1403.

Systemic hypoxia (*i.e.*, a decrease in arterial blood oxygen level) occurs under many different circumstances and affects a variety of physiologic systems. Systemic hypoxia can be either continuous or intermittent. Chronic continuous hypoxia is encountered during high-altitude sojourns, whereas chronic intermittent hypoxia (CIH) is a common and life-threatening condition that occurs in many different diseases, including sleep-disordered breathing, manifested as recurrent apneas. Recurrent apneas are characterized by repetitive, transient cessations of breathing (~10–30 sec), which result in periodic decreases in arterial blood oxygen. Nearly 50% of premature infants (32), 5% of middle-aged men, and 2% of women after menopause (25, 42) are prone to recurrent apneas. Physiologic systems adapt to chronic continuous hypoxia, whereas CIH associated with recurrent apneas results in autonomic abnormalities, alterations in sleep behavior, and impaired cognitive functions. Although arterial blood oxygen decreases in both continuous and intermittent hypoxia, it remains unclear why CIH results in morbidity.

Free radicals of oxygen are often referred to as reactive oxygen species (ROS), wherein an oxygen atom is much more reactive than molecular O_2. A variety of ROS have been identified, and their cellular sources of generation have been extensively investigated (for a review, see ref. 9). The biologic actions of ROS, especially superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$) are well docu-

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mented. ROS have been identified as one of the causative factors in a variety of morbidities. In this article, we present a brief overview of recent experimental models of CIH and clinical studies on recurrent apnea patients implicating a critical role for ROS in evoking phenotypic adverse effects.

ROLE OF ROS IN CIH-INDUCED FUNCTIONAL PLASTICITY IN THE CAROTID BODY AND VENTILATION

Hypoxia within seconds after its onset leads to stimulation of breathing and blood pressure. These compensatory responses to hypoxia are mediated entirely by reflexes arising from peripheral chemoreceptors, especially the carotid bodies. It has been proposed that carotid bodies constitute the "frontline" defense system for detecting systemic hypoxia associated with apneas (5). Studies on patients with recurrent apnea suggest that carotid body function is altered compared with that in control subjects (26). Direct recordings of the sensory activity demonstrate that CIH selectively augments the hypoxic but not hypercapnic sensory response of the carotid body (29–31, 35). In anesthetized rodents, acute intermittent hypoxia (AIH) increases sensory activity, which returns to baseline after terminating the stimulus. In striking contrast, in CIH-exposed animals, AIH leads to persistent increase in baseline activity even after terminating the stimulus. This long-lasting increase in baseline sensory activity of the carotid body has been termed sensory long-term facilitation [sensory LTF (30, 31)]. Thus, CIH, in addition to sensitizing the carotid body to hypoxia, induces a novel form of plasticity, which is manifested as the sensory LTF. The effects of CIH on the carotid body are time dependent (apparent after 3 days but not with 1 day of IH exposure) and can be reversed by normoxic reexposure for 10 days.

Activation of carotid body by hypoxia stimulates breathing. The hypoxic ventilatory response is augmented in patients with recurrent apnea (5) and in experimental animals exposed to CIH (31, 35), which can be attributed to enhanced carotid body sensitivity to hypoxia. Repetitive hypoxia leads to long-lasting activation of breathing (*i.e.*, LTF of breathing) (23), which is often referred to as plasticity of respiratory motor behavior. Interestingly, CIH enhances LTF of breathing (22, 28), an effect that can be attributed in part to the sensory LTF of the carotid body. Thus, CIH profoundly affects the hypoxic sensing ability of the carotid body and alters the ventilatory behavior to acute hypoxia.

Systemic administration of a membrane-permeable superoxide dismutase mimetic, MnTMPyP [manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride; 5 mg/kg/day for 10 days before CIH exposure], a potent scavenger of $O_2^{\cdot -}$, prevents the previously described effects of CIH on the carotid body (29, 30) and ventilation (28). Furthermore, CIH decreases aconitase enzyme activity [an index of $O_2^{\cdot -}$ production (8)] in carotid bodies, suggesting increased generation of ROS (30). The CIH-induced increase in ROS in the carotid body appears to arise in part from the inhibition of mitochondrial electron-transport chain (ETC) at complex I but not III (30). Taken together, these observations suggest that CIH activates ROS signaling pathways that contribute to altered carotid body function and the ensuing changes in breathing that may eventually lead to progression of apneas.

CIH-INDUCED CARDIOVASCULAR CHANGES AND THE ROLE OF ROS

Patients with recurrent apneas and experimental animals exposed to CIH exhibit elevated plasma catecholamines and increased blood pressures (3, 14, 34, 39). Bao *et al*. (3) reported that adrenelectomy prevents blood pressure elevations in CIH-exposed rats. A recent study (20) showed that CIH induces hypoxic sensitivity in the adrenal medulla of adult rats, which is otherwise relatively insensitive to acute low pO_2 (15, 43). These investigators showed that acute hypoxia evokes robust catecholamine efflux from the adrenal medulla of CIH-exposed rats, whereas low pO_2 is ineffective in eliciting similar effects in control rats. The effects of CIH are selective to hypoxia because either acidic or isohydric hypercapnia (10% CO_2) is ineffective in evoking catecholamine efflux from CIH adrenal medullae. The following lines of evidence suggest that ROS play a critical role in CIH-induced hypoxic sensitivity in the adrenal medulla. CIH significantly decreases aconitase enzyme activity both in the cytosolic and mitochondrial fractions, and the antioxidant MnTMPyP abolishes this effect, suggesting that CIH increases ROS levels in the adrenal medulla. More important, systemic administration of either MnTMPyP or *N*-acetylcysteine (NAC, a precursor for glutathione and a potent ROS scavenger) not only prevents CIH-induced hypoxic sensitivity in the adrenal medulla, but also abolishes CIH-induced blood pressure elevation and increases in plasma catecholamines (20). Thus, these observations suggest that ROS play a critical role in CIH-induced cardiovascular changes.

INVOLVEMENT OF ROS IN CIH-INDUCED CHANGES IN SLEEP AND COGNITIVE BEHAVIORS

Patients with obstructive sleep apneas (OSA) exhibit daytime hypersomnolence (37, 49). Despite therapy to alleviate OSA, many individuals exhibit considerable residual sleepiness during the day (6, 27). To assess whether intermittent hypoxia (IH) associated with apneas contributes to daytime sleepiness, Veasey *et al*. (45) examined sleep behavior in mice exposed to CIH. These authors found that in CIH-exposed mice, 2-h increase occurs in the total sleep time and a reduction in mean sleep latency relative to the control mice. The CIH-induced altered sleep behavior is associated with oxidative injury in basal forebrain and brainstem, as evidenced by elevated levels of isoprostane, protein carbonylation, increased nitration, and induction of glutathione reductase as well as methionine sulfoxide reductase A enzymes. Furthermore, CIH-induced oxidative injury in awake brain regions is associated with an increase in NADPH-oxidase gene and protein expression (52). More im-

portant, they (52) found that CIH-induced changes in sleep behavior as well as the oxidative injury are abolished by systemic administration of apocyanin, an inhibitor of NADPH oxidase, and is absent in gp91$^{phox-/-}$ mice with impaired NADPH oxidase function. These studies suggest that CIH *via* ROS generated by NADPH oxidase in brain regions associated with wakefulness contributes to altered sleep behavior.

Adult rats exposed to CIH exhibit significant impairments of spatial learning (36). Altered cognitive behavior is associated with increased apoptosis and oxidative injury, as evidenced by increased levels of malondialdehyde in the hippocampal CA1 region and cortex (16). Systemic administration of PNU-101033E, an antioxidant, not only abolishes the oxidative injury in these brain regions but also prevents CIH-induced cognitive impairment (36). Likewise, mice deficient in antioxidant apolipoprotein E exhibit increased cognitive impairment after CIH (16). These observations suggest that ROS signaling contributes to altered cognitive behavior in CIH-exposed rodents.

CIH ALTERS NEUROTRANSMITTER RESPONSES *VIA* ROS SIGNALING

Hypoglossal motoneurons regulate upper airway dilation involving serotonergic neurotransmission. Altered serotonergic transmission in hypoglossal motoneurons has been implicated in reduced upper airway dilation in experimental animals (12, 18). Clinical trials of serotonergic drugs in OSA patients, however, are ineffective (44). Responsiveness of hypoglossal motoneurons to serotonin is attenuated in CIH-exposed rats (46). The ineffectiveness of 5-hydroxytryptamine (5-HT)-receptor agonists is not due to reduction either in the number of hypoglossal motoneuron soma or in the expression of serotonergic postsynaptic receptor mRNA. Rather, it seems to be due to increased oxidative injury in the hypoglossal motoneurons, as evidenced by elevated isoprostane levels. Systemic administration of tempol (4-hydroxyl-2,2,6,6-tetramethylpiperidin-1-oxyl), an antioxidant, improves the responsiveness to serotonin and normalizes the medullary isoprostane levels in CIH-exposed rodents (46). These observations suggest that CIH-induced ROS, by altering the neuronal transmitter receptors, affects the hypoglossal motoneurons, which may contribute to the progression of OSA.

ROLE OF ROS IN CELLULAR RESPONSES TO INTERMITTENT HYPOXIA

Transcription of specific genes and the resulting *de novo* protein synthesis are considered critical for triggering adaptive responses to chronic hypoxia (40). Genes that are activated by hypoxia, in general, fall into two classes: (a) the immediate-early genes that are activated shortly after the onset of hypoxia, and (b) the late-response genes that are activated after several hours of hypoxia. Greenberg *et al*. (11) reported that CIH upregulates c-Fos, a member of the immediate-early gene family in the central nervous system. To examine the cellular mechanisms associated with *c-fos* upregulation by IH, Yuan *et al.* (50) developed a cell-culture model of IH wherein cells are exposed to alternating cycles of hypoxia, similar to the paradigm used in intact animals. By using this model, they found that IH elevates *c-fos* mRNA in a stimulus-dependent manner, increases the transcriptional activation of the *c-fos* gene, and augments the transcriptional activation of activator protein-1 (AP-1). Anti-sense *c-fos* abolishes IH-evoked AP-1 activation, suggesting the importance of *c-fos* upregulation in this response. Intriguingly, IH-induced *c-fos* activation persists for 3 h after terminating the IH stimulus. This persistent activation of *c-fos* is reminiscent of CIH-induced sensory LTF of the carotid body in intact animals, suggesting that an LTF-like phenomenon can be elicited by IH, even at the gene level. Interestingly, IH-evoked *c-fos* mRNA expression correlates to the duration of intervening normoxic rather than hypoxic episodes, suggesting that reoxygenation is the determining factor for evoking the early gene expression by IH. The effects of IH on *c-fos* mRNA and AP-1 are associated with markedly decreased aconitase activity in the cytosolic and mitochondrial fractions, indicating increased generation of ROS. Furthermore, these IH-evoked effects are prevented by pretreating the cells with MnTMPyP. These investigators (50) further showed that ROS generation is increased in the mitochondria isolated from IH-exposed PC12 cells, which is associated with the downregulation of complex I activity.

The transcriptional activator, hypoxia-inducible factor-1 (HIF-1), is considered a master regulator of a variety of genes during hypoxia (40). IH increases HIF-1α (the O_2-regulated subunit of the HIF-1 complex) as well as HIF-1–mediated transcriptional activation in a stimulus-dependent manner in PC12 cells (51). IH-induced increase in HIF-1–mediated transcriptional activity is Ca^{2+} dependent and requires calcium/calmodulin–dependent protein kinase II (CaMK II)-dependent phosphorylation of p300 coactivator (51). To assess the physiologic significance of HIF-1 activation Peng *et al.* (31) examined the effects of CIH on *Hif1a*$^{+/-}$ mice. These investigators found that CIH resulted in augmented carotid body response to hypoxia and induced sensory LTF of the chemoreceptor activity in CIH-exposed wild-type mice, but not in CIH-exposed *Hif1a*$^{+/-}$ mice. Further analysis of cardiorespiratory responses revealed augmented hypoxic ventilatory response, LTF of breathing, elevated blood pressures, and increased plasma norpeipnephrine in CIH-exposed wild-type mice, and these responses were either absent or attenuated in CIH-exposed *Hif1a*$^{+/-}$ mice. In CIH-exposed wild-type mice, ROS were elevated, and MnTMPyP prevented this response. Intriguingly, ROS levels were unaltered in response to CIH in *Hif1a*$^{+/-}$ mice, suggesting complex positive interactions between HIF-1 and ROS generation (31, 41).

IH also increases tyrosine hydroxylase (TH) enzyme activity (the rate-limiting enzyme in catecholamine synthesis) in PC12 cells, and this effect is mediated by increased serine phosphorylation involving activation of protein kinase A as well as calcium/calmodulin-dependent protein kinase (19). These studies suggest that IH stimulates transcriptional as well as post-translational mechanisms.

In addition to its effects on gene and protein expression, IH also affects transmitter secretion from PC12 cells. IH is shown

to potentiate hypoxia-evoked transmitter release and induce the release of transmitters that are not normally evoked by hypoxia. For instance, hypoxia-evoked dopamine (DA) release is augmented by IH, whereas the release of acetylcholine (ACh), which is not facilitated by acute hypoxia, is induced after IH exposure (17). The facilitatory and inductive effects of IH on hypoxia-evoked DA and ACh release, respectively, is blocked by 2-APB (2-aminoethoxydiphenylborate), a blocker of inositol 1,4,5-triphosphate ($InosP_3$) receptors, but not by voltage-gated Ca^{2+}-channel inhibitors (17). Conversely, 2-APB had no obvious effects on transmitter release in control cells, suggesting that IH recruits intracellular Ca^{2+} mobilization pathways for stimulus-evoked transmitter release. Recently, Wang *et al.* (47) reported that IH stimulates brain-derived neurotrophic factor (BDNF) release from neuronally differentiated PC12 cells. IH-induced BDNF release requires activation of tetrodotoxin-sensitive Na^+ channels and Ca^{2+} influx through N- and L-type Ca^{2+} channels, as well as mobilization of internal Ca^{2+} stores. More important, the effects of IH on BDNF release can be prevented by antioxidants (MnTMPyP or NAC). These observations suggest that IH *via* ROS-dependent signaling recruits intracellular Ca^{2+} mobilization pathways for facilitating stimulus-evoked secretion of neurotransmitters and neurotrophic factor.

Undifferentiated PC12 cells resemble glomus cells of the carotid body with regard to their sensitivity to acute hypoxia. Release of neurotransmitters from the glomus cells is important for sensory transmission at the carotid body (33). Therefore, IH-evoked facilitation of transmitter release might be of functional importance in the altered carotid body function. BDNF, conversely, is known to be essential for neuronal survival. As cited in the preceding section, IH induces neuronal apoptosis (16). A recent study reported that BDNF plays a role in IH-induced LTF of breathing (1). It is possible that the facilitated BDNF secretion may be beneficial in preventing the deleterious effects of IH in the central neurons and can participate in LTF of breathing. These possibilities, however, require further studies.

ELEVATED ROS IN PATIENTS WITH RECURRENT APNEA

Studies outlined thus far suggest that IH increases ROS in experimental animals and in cell cultures. A number of clinical studies reported that the levels of several biomarkers of oxidative injury are elevated in the body fluids, breath, and cells derived from humans experiencing CIH as a consequence of recurrent apneas (Table 1). Dyugobskaya *et al.* (7) reported an increase in ROS generation in CD11C-positive monocytes derived from OSA patients, and ROS seem to contribute to upregulation of adhesion molecules (CD15 and CD11C) in monocytes and increased adhesion to endothelial cells. These effects are reversed after nasal continuous positive airway pressure (CPAP) treatment. A recent study by Grebe *et al.* (10) reported

Table 1. Evidence for Oxidative Stress in Obstructive Sleep Apnea (OSA) Patients

Type of apnea	*Biomarkers of oxidative injury*	*Source of measurement*	*Increase/ decrease*	*Effect of CPAP therapy*	*Reference*
OSA	Glutathione peroxidase, homocysteine, vitamin B_{12}, folate	Plasma	No change		(2)
	Vitamin A		Decreased	Normalized	
	Vitamin E		Increased	Normalized	
	γ-Glutamyl transferase				
OSA	Malondialdehyde	Plasma	Increased	Normalized	(13)
	O, *O*′-dityrosine	Urine	Increased	Normalized	
OSA	8-hydroxy-2′-deoxyguanosine	Urine	Increased	ND	(48)
OSA	TBARS	Plasma	Increased	Normalized	(21)
	Peroxides				
	Antioxidant protective enzyme (PON1)		Decreased		
OSA	8-Isoprostane	Exhaled breath condensate	Elevated	Decreased	(4)
OSA	Superoxide anion	Neutrophils	Elevated	Decreased	(38)
OSA	ROS	Monocytes	Elevated	Decreased	(7)

ND, not done.

that flow-mediated dilation of the brachial artery is reduced in OSA patients, which could be restored by systemic administration of vitamin C, suggesting that oxidative stress is responsible for endothelial dysfunction in OSA patients. However, few studies exist wherein absence of evidence for oxidative injury in patients with recurrent apnea is reported. For instance, Montgomery-Downs *et al.* (24) were unable to detect changes in the levels of urinary F(2)-isoprostane metabolites, a marker of ROS generation in children with sleep-disordered breathing. Jordan *et al.* (13), however, pointed out that the prevailing conflicting result on ROS levels in recurrent apnea patients as compared with the control subjects may arise from methodologic difficulties, as well as gender differences. Consequently, for reliable assessment of oxidative stress, these investigators (13) recommend simultaneous measurement of multiple biomarkers of oxidative injury at different time intervals during sleep and wakefulness.

In summary, studies described thus far show that CIH exerts a variety of systemic and cellular responses not only in experimental models but also in recurrent apnea patients. It is intriguing how CIH, despite comprising brief and modest severity of hypoxia, is able to evoke a range of robust and long-lasting systemic and cellular responses. One likely explanation is that ROS, which are the metabolites of molecular O_2, play a novel role as amplifiers of brief hypoxic signals associated with IH in that they, *via* activation of specific transcriptional responses and signaling pathways, contribute to the effective translation of the hypoxic signals, leading to systemic responses associated with recurrent apneas, as illustrated in Fig. 1. Although experimental studies identified that inhibition of the mitochondrial electron-transport chain at complex I and activation of NADPH oxidase potentially contributes to ROS generation by IH, the underlying mechanisms remain to be investigated. Antioxidants not only prevent many of the CIH-evoked physiologic and cellular responses in experimental settings, but more important, they offer protection against certain phenotypic adverse effects in apnea patients (10). These findings emphasize the potential therapeutic value of antioxidants to alleviate certain morbidities associated with recurrent apneas.

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ABBREVIATIONS

AIH, acute intermittent hypoxia; AP-1, activator protein-1; 2-APB, 2-aminoethoxydiphenylborate; BDNF, brain-derived neurotrophic factor; CaMK II, calcium/calmodulin-dependent protein kinase-II; CIH, chronic intermittent hypoxia; CPAP, continuous positive airway pressure; ETC, electron-transport chain; 5-HT, 5-hydroxytryptamine; HIF-1, hypoxia-inducible factor-1; IH, intermittent hypoxia; $InosP_3$, inositol 1,4,5-triphosphate; LTF, long-term facilitation; MnTMPyP, manganese III tetrakis (1-methyl-4-pyridyl)-porphyrin pentachloride; NAC, *N*-acetyl cysteine; OSA, obstructive sleep apnea; Po_2, partial pressure of oxygen; ROS, reactive oxygen species; TBARS, thiobarbituric acid–reactive substances; TH, tyrosine hydroxylase.

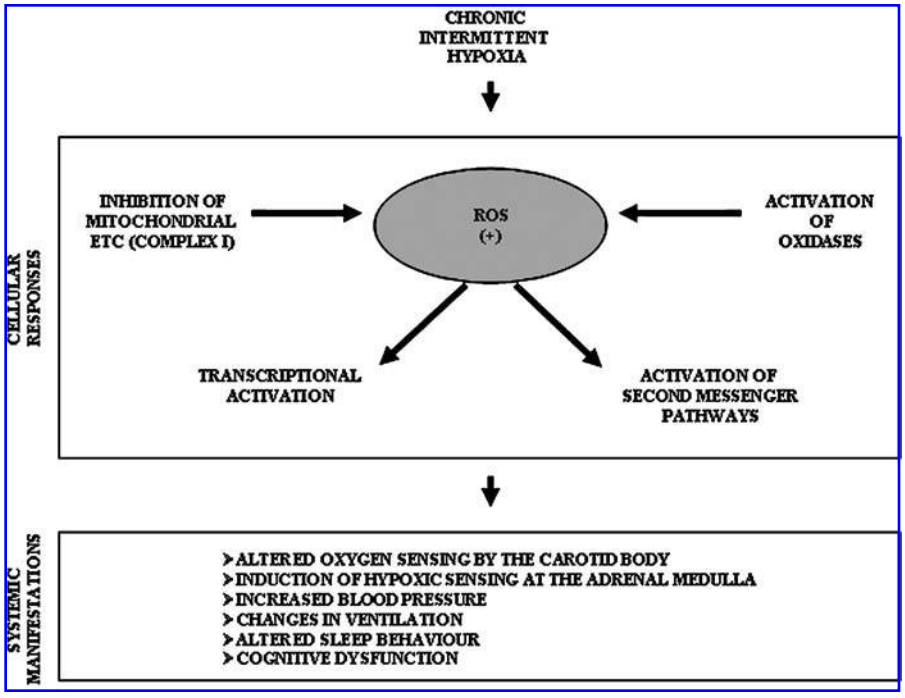

FIG. 1. Schematic illustration of cellular responses and subsequent systemic manifestations in response to chronic intermittent hypoxia. ROS, reactive oxygen species; ETC, electron-transport chain.

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