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SENSORY PLASTICITY OF THE CAROTID BODY: ROLE OF REACTIVE OXYGEN SPECIES AND PHYSIOLOGICAL SIGNIFICANCE

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Abstract

Recent studies have shown that acute intermittent hypoxia (IH) induces sensory plasticity of the carotid body manifested as sensory long-term facilitation (LTF), which requires prior conditioning with chronic IH and is mediated by reactive oxygen species (ROS). The purpose of this article is to provide a brief review of chronic IH-induced sensory LTF of the carotid body, sources of ROS, mechanisms underlying sensory LTF and its functional significance. Development of sensory LTF requires conditioning with several days of chronic IH. It is completely reversible following re-oxygenation, does not depend on the severity of hypoxia used for IH conditioning, not species specific and is selectively evoked by acute repetitive hypoxia but not by repetitive hypercapnia. Sensory LTF is not associated morphological changes in the carotid body and is expressed in chronic IH treated adult but not in neonatal rat carotid bodies. Chronic IH increases ROS levels in the carotid body involving 5-HT mediated activation of NADPH oxidase-2 (NOX2) and subsequent inhibition of the mitochondrial complex I. Sensory LTF can be prevented by inhibitors of 5-HT receptors, NOX inhibitors as well as by anti-oxidants. The signaling pathways mediating the sensory LTF are summarized in figure 2. It is suggested that sensory LTF contributes to the persistent sympathetic excitation under chronic IH.

Keywords

sensory plasticity; recurrent apneas; neurotransmitters; H₂O₂; superoxide anion; HIF-1; HIF-2; Pet-1 transcription factor

1. Introduction

Stimulation of breathing by hypoxia is a reflex and arterial chemoreceptors, especially the carotid body constitute the major afferent limb of this reflex. It is increasingly being appreciated that ventilatory responses to hypoxia are not hard wired rather display considerable plasticity. A well documented plasticity of the respiratory motor activity is its response to acute intermittent hypoxia (IH). Following acute IH, respiratory moto-neuron

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activity exhibits long-lasting activation which persist even after termination of the stimulus---a phenomenon termed as long-term facilitation (LTF) of breathing (see Powell *et al.*, 1998). Respiratory LTF is unique to acute IH and is not evident with continuous hypoxia (Baker and Mitchell, 2000).

Until recently it was not known whether acute IH also induces a similar plasticity at the afferent limb of the chemoreflex i.e., the carotid body. Studies by Peng *et al* (2003; 2006b) demonstrated for the first time that plasticity of the carotid body, manifested as long-lasting sensory excitation or sensory LTF, can be induced by repetitive hypoxia. However, unlike the LTF of breathing, sensory LTF of the carotid body requires prior conditioning with chronic IH. It is being increasingly recognized that reactive oxygen species (ROS) which represent free radicals of oxygen, function as signaling molecules in a variety of physiological systems. There is considerable evidence implicating ROS signaling as a critical mediator of sensory LTF (Prabhakar *et al.*, 2007). The purpose of this article is to provide a brief review of chronic IH-induced sensory LTF of the carotid body, sources of ROS, and mechanisms by which ROS mediate this phenomenon, as well as the significance of sensory LTF in regulating autonomic functions under chronic IH.

2. Chronic intermittent hypoxia (IH)-induces sensory long-term facilitation (LTF) of the Carotid Body

When anesthetized rats were challenged with acute IH (10 episodes of 15 sec of 12% O₂ interspersed with 5 min of 95% O₂), the sensory activity increased with each episode of hypoxia and promptly returned to baseline value after terminating the 10th hypoxic episode (Peng *et al.*, 2003). A study by Cummings and Wilson (2005) reported that acute IH causes a modest increase in baseline sensory activity in an *in vitro* perfused rat carotid body preparation, and this effect was elicited only with modest intermittent hypocapnic hypoxia.

On the other hand, when rats were treated chronically with IH (15 sec of 5% O₂ interspersed with 5 min of normoxia; 8h/day for 10 days) and then challenged with acute IH there was a robust increase in baseline carotid body activity, which continued to increase further during 60min of the post-acute IH period (Fig.1). This increase in baseline sensory activity, observed in *in vivo* carotid body preparation, occurred despite maintaining arterial blood gas composition and blood pressure comparable to control pre-acute IH period (Peng *et al.*, 2003). The time course of the increase in carotid body sensory activity resembled that of LTF of breathing elicited by acute IH reported previously (Mitchell and Johnson, 2003) and hence was referred as sensory LTF. These observations demonstrate that sensory LTF is either not induced or induced only weakly in control carotid bodies; whereas it can be robustly induced by acute IH following chronic IH.

2.1. Characterization of sensory LTF

Peng *et al* (2003) noted following characteristics of sensory LTF in chronic IH treated rat carotid bodies. *First*, it developed overtime and was not seen in rats treated with 1 day of IH, but appeared after 3 days of IH and further increased in magnitude following 10 days of IH. *Second*, sensory LTF was reversible. When rats treated with 10 days of IH were placed in room air for 3 days (i.e., re-oxygenation), the magnitude of sensory LTF was reduced by ~40%, and it was completely absent following 10 days of re-oxygenation. *Third*, the magnitude of sensory LTF was independent of the severity of hypoxia used for IH conditioning. For instance, the magnitude of sensory LTF remained the same in rats conditioned with IH everyday using either 10% O₂ (mild hypoxia) or 5% O₂ (severe hypoxia). *Fourth*, sensory LTF was not species specific as it can be seen in rats (Peng *et al.*, 2003) as well as mice (Peng *et al.*, 2006b), two species studied thus far. *Fifth*, sensory

LTF was selective to the hypoxic stimulus, because it was not elicited by acute intermittent hyperoxic hypercapnia (10 episodes of 15 sec of 7% CO₂ + 93% O₂ interspersed with 5 min of 100% O₂) in chronic IH treated carotid bodies. These observations demonstrate that chronic IH-induced sensory LTF is a time-dependent phenomenon, completely reversible following re-oxygenation, does not depend on the severity of hypoxia used for IH conditioning, not species specific and is selectively evoked by acute repetitive hypoxia but not by repetitive hypercapnia.

2.2. Induction of sensory LTF depends on age

Depending on gestational age, nearly 90% of prematurely born infants experience IH as a consequence of recurrent apneas (Poets *et al.*, 1994). Carotid bodies are immature at birth and respond poorly to hypoxia compared with adults (Blanco *et al.*, 1984, Donnelly., 2000, Carroll., 2003). However, pre-term infants exhibiting greater number of apneas display augmented peripheral chemoreceptor reflex as evidenced by more pronounced ventilatory depression by brief hyperoxia (Dejour's test) than those having lesser incidence of apneas (Nock *et al.*, 2004, Cardot *et al.*, 2007).

Given that pre-term infants experience chronic IH, Pawar *et al.* (2008) examined sensory LTF in neonatal rat model of chronic IH. In this study rat pups were treated with IH from ages P0-P10. In striking contrast to adults, acute IH was ineffective in eliciting sensory LTF in neonatal carotid bodies. However, neonatal carotid bodies treated with chronic IH exhibited heightened hypoxic sensitivity, similar to that seen in adults (Pawar *et al.*, 2008, Peng and Prabhakar, 2004, Rey *et al.*, 2004), suggesting that the absence of sensory LTF was not due to the lack of the effects of chronic IH on the sensory activity of the carotid body. Also, these findings indicate that heightened hypoxic sensitivity caused by chronic IH is not causally related to sensory LTF. These findings suggest that age is an important factor in inducing sensory LTF of the carotid body.

2.3 Dependence of sensory LTF on pattern of IH conditioning

The pattern of chronic IH employed in the above described studies is akin to that seen during sleep disordered breathing (SDB) with apneas (either obstructive or central apneas), wherein the duration of each hypoxic episode, depending on the length of apneas, lasts between 10 and 40 s, and interspersed with normoxic periods that last minutes. However, other patterns of IH have been described (see Prabhakar 2001). One such other pattern of IH is that encountered during rapid ascent and descent from high altitude as well as in patients with chronic lung disease, wherein ventilation is depressed during sleep and each episode of hypoxia lasts between several minutes to hours. To assess whether this pattern of IH also induces sensory LTF, rats were treated with either a single episode of 4h of hypoxia followed by 20h of normoxia or 4h of hypoxia per day for 10 days. Conditioning with this pattern of IH either acutely or chronically did not elicit sensory LTF (Peng *et al.*, 2003). These findings demonstrate that induction of sensory LTF critically depends on the paradigm of IH used for conditioning.

2.4. Sensory LTF in *ex vivo* carotid bodies

Sensory LTF might be secondary to cardiovascular changes affecting vascular perfusion of the carotid bodies. Therefore, studies were performed on *ex vivo* superfused carotid body preparations wherein vascular effects are effectively absent. In response to acute IH sensory LTF could readily be evoked in chronic IH but not from normoxia treated control rat (Peng *et al.*, 2003) and mouse carotid bodies (Peng *et al.*, 2006b). These observations suggest that sensory LTF is due to direct effects of chronic IH on glomus tissue rather than secondary to vascular changes in the carotid body.

3. Mechanisms underlying the induction of sensory LTF

Carotid body sensory response to hypoxia depends on the morphology of glomus tissue as well as the transduction cascade involving a variety of K^+ channels eventually leading to Ca^{2+} -dependent release of neurotransmitter(s) from glomus cells (Prabhakar, 2000). The following section summarizes the effects of chronic IH on carotid body morphology and the role of transmitter(s) in mediating sensory LTF.

3.1. Carotid body morphology

Chronic continuous hypoxia leads to hyperplasia of the glomus cells and hypertrophy as well as neo-vascularization in the carotid body (Laidler and Kay., 1978, Pequignot *et al.*, 1984, Dhillon *et al.*, 1984, Heath *et al.*, 1985; Jyung *et al.*, 2000). To assess the impact of chronic IH on carotid body morphology morphometric analysis was performed. Unlike chronic continuous hypoxia, chronic IH treated carotid bodies showed no apparent differences in the total volume of the carotid body, number of glomus cells, or glomus cell volume compared to controls (Peng *et al.*, 2003). These findings suggest that sensory LTF is unlikely due to changes in carotid body morphology.

3.2. 5-Hydroxytryptamine (5-HT) and sensory LTF

Among the various neurotransmitters, 5-HT has been implicated in long-term neuronal activation in the vertebrate and invertebrate nervous system. For instance, repetitive applications of 5-HT evoke long-term potentiation (LTP) in the Aplysia nervous system (Mauelshagen *et al.*, 1998, Michael *et al.*, 1998). In vertebrates, 5-HT mediates long-lasting activation of spinal reflexes evoked by repetitive stimulation of nociceptive afferents (Machacek *et al.*, 2001). 5-HT is also critical for eliciting LTF of the respiratory motor activity evoked by repetitive hypoxia (Mitchell and Johnson, 2003, Bocchiario and Feldman, 2004).

5-HT is expressed in the carotid body (Gronblad *et al.*, 1983; Habeck *et al.*, 1994; Zhang *et al.*, 2003; Jacono *et al.*, 2005) and exogenous application of 5-HT stimulates the carotid body activity (Nishi, 1975; Kirby and McQueen., 1984; Jacono *et al.*, 2005). Although the role of 5-HT in carotid body response to acute hypoxia is uncertain, it might mediate sensory LTF as it does elsewhere in the nervous system. If 5-HT is involved in sensory LTF of the carotid body then it should satisfy the following criteria: a) it should be released in response to hypoxia; b) exogenous repetitive but not bolus administration of 5-HT should evoke sensory LTF; c) sensory LTF should be absent in carotid bodies lacking 5-HT; and d) blockade of 5-HT receptors should prevent sensory LTF.

Recent studies provide evidence for each of the above criteria implicating 5-HT as an important transmitter mediating sensory LTF. First, acute hypoxia releases 5-HT from chronic IH treated carotid bodies but not from control glomus tissue and this response seem to involve mobilization of intracellular Ca^{2+} stores (Peng *et al.*, 2009). Second, repetitive applications of 5-HT (3×15 s of 100 nM at 5 min intervals) but not continuous application (300 nM, 45 s) evoked sensory LTF in control carotid bodies (Peng *et al.*, 2006a). Third, mice deficient in the gene encoding the Pet-1 transcription factor exhibited markedly reduced 5-HT expression in the glomus cells and showed remarkable absence of sensory LTF in chronic IH treated Pet-1^{-/-} mice (Peng *et al.*, 2009). Fourth, ketanserin, a 5-HT₂ receptor antagonist prevented chronic IH-induced sensory LTF (Peng *et al.*, 2009) as well as that evoked by exogenous applications of 5-HT (Peng *et al.*, 2006a). These observations suggest that 5-HT release from glomus cells and subsequent activation of 5-HT₂ receptors are critical for evoking sensory LTF by chronic IH.

3.2.1. 5-HT mediates sensory LTF via reactive oxygen species (ROS) signaling

3.2.1.1. Critical role for ROS in evoking sensory LTF: Reactive oxygen species (ROS) have been implicated in long-term potentiation of neural activity (Kishida *et al.*, 2006, Kishida and Klann, 2007). ROS signaling mediates many of the cellular and systemic responses to chronic IH (Prabhakar *et al.*, 2007). Biochemical studies showed that chronic IH increases ROS levels in the carotid body and anti-oxidant treatment prevented the induction of sensory LTF in chronic IH treated rats (Peng *et al.* 2003).

Free radicals of oxygen, such as $O_2^{\cdot-}$, H_2O_2 , and $OH^{\cdot-}$ constitute ROS. The following observations from Peng *et al.* (2009) suggest that H_2O_2 generated from $O_2^{\cdot-}$ contributes to sensory LTF. Acute IH-induced sensory LTF was absent in carotid bodies in presence of continuous application of MnTMPyP, a membrane permeable scavenger of $O_2^{\cdot-}$. However, when MnTMPyP was applied 15 min after the onset of sensory LTF, it did not prevent further development of sensory LTF; whereas substituting MnTMPyP with PEG-catalase, a membrane permeable scavenger of H_2O_2 did prevent the development of sensory LTF. These observations suggest that H_2O_2 generated from $O_2^{\cdot-}$ mediates sensory LTF. A role for H_2O_2 in eliciting sensory LTF was further supported by the following observations. When normoxia treated carotid bodies were superfused for 90 min with H_2O_2 as little as 500 nM, acute IH evoked robust sensory LTF similar to that seen with chronic IH treated carotid bodies; whereas H_2O_2 by itself had no effect on the baseline sensory activity when applied for 90 min.

3.2.1.2. 5-HT activates NADPH oxidase (NOX): The studies outlined above suggest that H_2O_2 generated from $O_2^{\cdot-}$ as well as 5-HT mediate sensory LTF. Is there a cross-talk between 5-HT and the mechanisms underlying $O_2^{\cdot-}$ generation? NADPH oxidases (NOX) constitute major cellular sources of $O_2^{\cdot-}$. NOX family of oxidases include NOX1, 2, 3, and 4 isoforms (see Bedard and Krause., 2007). Peng *et al.* (2009) examined role of NOX in sensory LTF of the carotid body. They found that NOX2 mRNA and protein were upregulated in chronic IH treated carotid bodies and the protein was localized to the cytoplasm of glomus cells. It was further shown that despite the up-regulation of NOX2 protein, basal NOX activity in chronic IH treated carotid bodies was the same as controls, suggesting that the NOX enzyme was not constitutively active. Following acute IH NOX enzyme activity was markedly increased (~12 fold) and this effect was seen in chronic IH treated but not in control carotid bodies. Ketanserin, a 5-HT₂ receptor antagonist prevented acute IH- induced NOX activation (Peng *et al.*, 2009), suggesting 5-HT mediates NOX activation by acute IH. Repetitive applications of 5-HT increased NOX activity in the carotid body and this response required protein kinase C (PKC) activation by 5-HT₂ receptors (Peng *et al.*, 2006a). These findings further support a role for 5-HT in mediating acute IH-induced NOX activation. Pharmacological inhibitors of NOX (e.g., apocynin, DPI and AEBSF) prevented the sensory LTF in chronic IH treated rats (Peng *et al.*, 2009) as well as that evoked by repetitive application of 5-HT (Peng *et al.*, 2006a). Furthermore, sensory LTF was absent in mice deficient in gp91^{phox}, the catalytic subunit of the NOX2 complex elicited by either chronic IH (Peng *et al.*, 2009) or with repetitive applications of 5-HT (Peng *et al.*, 2006a). These observations suggest that 5-HT-induced NOX2 activation and the ensuing $O_2^{\cdot-}$ are critical for evoking sensory LTF.

3.2.1.3. NOX2 mediates inhibition of mitochondrial complex I activity: Evidence for ROS-induced ROS: Mitochondrial electron transport chain (ETC) complexes are another major source of ROS (Ambrosio *et al.*, 1993). Analysis of ETC complex activity showed that the activity of the complex I but not that of complex III was markedly inhibited in chronic IH treated carotid bodies (Peng *et al.*, 2003). The following findings from rat pheochromocytoma 12 (PC12) cell cultures and central nervous system (Khan *et al.*, 2011)

demonstrate that NOX2 mediates IH-induced complex I inhibition: a) Inhibition of NOX by apocynin or AEBSF as well as loss of NOX function by silencing RNA approach prevented complex I inhibition by IH and b) complex I inhibition was absent in IH treated NOX2 (gp 91^{phox}) knockout mice. This study further showed that ROS generated by NOX2 facilitates Ca²⁺ influx into mitochondria and causes oxidative modification of complex I as evidenced by S-glutathionylation of 75- and 50-kDa subunits which leads to inhibition of the complex I activity. The decreased complex I activity was associated with increased mitochondrial ROS (Yuan *et al.*, 2004; Khan *et al.*, 2011). These observations provide evidence for a cross talk between membrane bound NOX2 and the mitochondrial complex I leading to ROS-induced ROS (positive feed-forward mechanism), which contributes to sensory LTF. Figure 2 illustrates signaling mechanisms contributing to the chronic IH-induced sensory LTF of the carotid body.

4. Is 5-HT the sole mediator of sensory LTF of the carotid body?

Besides 5-HT, carotid bodies express a variety of other excitatory modulators including acetylcholine (ACh), adenosine triphosphate (ATP), substance P (SP) and angiotensin II (AT-II) (Prabhakar, 2000). A recent study examined the effects of repetitive application of ACh, ATP, SP and AT-II as well as KCl, a non-selective depolarizing agent (Peng *et al.*, 2011b). Although ACh, ATP, SP, and KCl stimulated the carotid body activity with each application, they did not induce sensory LTF. In striking contrast, repetitive application of Ang II as little as 60 pM with each application (5 x every 30s at 5 min intervals) caused modest excitation of the carotid body and induced robust sensory LTF similar to that seen with repetitive application of 5-HT (Peng *et al.*, 2006a). AT-II-induced sensory LTF is unlikely due to vascular changes because it was seen in superfused carotid bodies, wherein vascular influences of AT-II are effectively absent. Furthermore, Ang II is a more potent inducer of sensory LTF than 5-HT because it can be elicited with picomolar concentrations of Ang II as opposed to nanomolar concentration of 5-HT needed for sensory LTF.

The following observations by Peng *et al.* (2011b) further demonstrate that AT-II-induced sensory LTF involves NOX2/ROS signaling: a) repetitive application of Ang II increased ROS in the carotid body; b) apocynin and AEBSF, two known inhibitors of Nox2 blocked Ang II-induced ROS generation as well as sensory LTF; and c) Ang II-evoked sensory LTF was absent in mice deficient in gp91^{phox}, the catalytic subunit of Nox2. Losartan, an established AT-1 receptor antagonist also prevented Ang-II evoked sensory LTF as well as ROS generation, suggesting that AT-II induced NOX2 activation is mediated by AT-1 receptors. The observations that both 5-HT and Ang II elicit sensory LTF via NOX2/ROS signaling raise an intriguing possibility that chronic IH-induced sensory LTF requires interaction between serotonergic and angiotensinergic systems in that 5-HT might facilitate Ang II release or vice versa. These possibilities, however, remain to be investigated.

5. Molecular mechanisms underlying sensory LTF: Role of hypoxia-inducible factors (HIFs)

The long-term effects of hypoxia require activation of specific transcription factors and the ensuing alterations in gene expression. Chronic IH activates a variety of transcription factors, including the hypoxia-inducible factors (HIFs), *c-fos* (immediate early gene), nuclear factor of activated T-cells (NFAT), and nuclear factor κ B (NF- κ B) (see Nanduri *et al.*, 2008). The role of HIFs in sensory LTF was examined because the effects of IH on other transcription factors are cell-type and cell-state specific.

HIF-1, the prototypical member of the HIF family comprises an O₂-regulated subunit and a constitutive β subunit (Semenza, 2000). Chronic IH activates HIF-1 mediated transcription

(Yuan *et al.*, 2005, 2008). The role of HIF-1 in sensory LTF was examined in *Hif1a*^{+/-} heterozygous (HET) mice because complete HIF-1 α deficiency results in embryonic lethality at mid-gestation. Chronic IH treated *Hif1a*^{+/-} mice exhibited remarkable absence of sensory LTF and unaltered ROS levels; whereas these phenotypic responses were seen in wild type mice treated with chronic IH (Peng *et al.*, 2006b). How might HIF-1 contribute to sensory LTF? A recent study showed that transcriptional up-regulation of NOX2 in chronic IH treated carotid bodies requires HIF-1 activation (Yuan *et al.*, 2011). It is conceivable that the absence of NOX2 up-regulation and ROS generation might in part account for the lack of sensory LTF in *Hif1a*^{+/-} mice.

HIF-2 α (also known as endothelial PAS domain protein-1, EPAS-1) is another member of the HIF family (Tian *et al.*, 1997), which shares ~50% sequence homology to HIF-1 α and also interacts with HIF-1 β (Ema *et al.*, 1997). Continuous hypoxia activates HIF-1- and HIF-2-mediated transcriptions, which in turn regulate several common target genes (Holmquist-Mengelbier *et al.*, 2006). Nanduri *et al.* (2009) reported that in contrast to continuous hypoxia, IH down regulates HIF-2 α protein in the carotid body and this effect is mediated by Ca²⁺-dependent protease, calpain. These investigators further showed that IH-induced HIF-2 α degradation results in transcriptional down of *Sod-2* mRNA, which encodes the anti-oxidant enzyme, superoxide dismutase-2. Furthermore, mRNAs encoding several anti-oxidant enzymes including the *Sod-2* were down regulated in the carotid bodies from *Hif2a*^{+/-} mice and the phenotypic responses of these mice were similar to chronic IH treated mice (Peng *et al.*, 2011a). These observations suggest that HIF-2 contributes to IH-induced elevation of ROS via decreased transcription of anti-oxidant enzymes. Thus, it appears that the imbalance between HIF-1 and HIF-2 contributes to chronic IH-induced sensory LTF via transcriptional up regulation of pro-oxidants (e.g., NOX2) by HIF-1 and down regulation of anti-oxidant enzymes (e.g., SOD-2) by HIF-2 resulting in increased ROS levels.

6. Physiological Significance of Sensory LTF of the Carotid Body

It is increasingly being appreciated that altered carotid body function and the ensuing changes in chemo-reflex responses contribute to morbidities of the autonomic nervous system associated with many pathological conditions (Prabhakar and Peng, 2004, Schulz and Li., 2007). Adult humans with recurrent apneas experience chronic IH and exhibit increased sympathetic nerve activity not only with each episode of apnea, but also during the day, even in the absence of apnoeas. There is considerable evidence suggesting that peripheral chemo-reflex function is augmented in recurrent apnea patients (Narkiewicz *et al.*, 1998). It is conceivable that IH resulting from recurrent apneas leads to sensory LTF of the carotid body, which in turn triggers reflex activation of sympathetic nervous system. Such a possibility might account for the persistent day time sympathetic excitation seen in these subjects. Chemo-reflexes from the carotid body are implicated in causing persistent sympathetic excitation in other diseases such as congestive heart failure (CHF; Schulz and Li., 2007). Whether sensory LTF of the carotid body is also expressed in CHF and it contributes to persistent activation of sympathetic nervous system remains to be explored.

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FIGURE. 1.

Examples illustrating the effects of acute intermittent hypoxia (IH at arrows) on sensory activity of the *ex vivo* carotid bodies from control (i.e., normoxia treated) and CIH (chronic IH) treated adult, male, Sprague-dawley rats. Insets represent “Single” fibers from which the data was derived. Note the long-lasting increase in baseline activity following the termination of acute IH in CIH but not in control. This long-lasting increase in sensory activity is referred as sensory long-term facilitation (LTF).

FIGURE 2.

Schematic illustration of signaling mechanisms associated with the induction of sensory LTF of the carotid body by chronic IH. CIH= chronic intermittent hypoxia; CB= carotid body; 5-HT= 5-hydroxytryptamine; Ang II= angiotensin II; ROS= reactive oxygen species; Sensory LTF= sensory long-term facilitation.