Differential expressions and roles of hypoxia-inducible factor-1α, -2α and -3α in the rat carotid body during chronic and intermittent hypoxia

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Summary. The HIF-1α expression in the carotid body (CB) is central to the transcriptional regulation of the CB structural and functional changes in chronic hypoxia (CH). The CB plays pathogenic roles in cardiovascular morbidity in patients with sleep-disordered breathing; yet, the expression and role of HIF-α subtypes in intermittent hypoxia (IH), resembling recurrent episodic apnea, are unclear. We hypothesized a divergent role of HIF-α subtypes, regulated by differential expression in the CB response to IH. A time-course analysis of the CB volume, and expression profiles of the HIF-1α, -2α, -3α and HIF-regulated gene products, including vascular endothelial growth factor (VEGF), endothelin-1 (ET-1), and tyrosine hydroxylase (TH), showed a significant difference in the lack of increase in the rat CB volume, HIF-1α and VEGF expression during IH, despite an increase in the mRNA level of HIF-1α and the prominent increase of volume and expression in the CH group. In contrast, there were increased CB expressions of HIF-2α and -3α, and also ET-1 and TH in both IH and CH groups. Results demonstrated a significant role played by HIF-2α and -3α in the CB response to IH, which could be complementary to the expression and role of HIF-1α under hypoxic conditions. This differential regulation of the HIF-α subtypes and pathways could account for the morphological and neurochemical discrepancy in the CB responses to IH and CH.

Key words: Hypoxia-inducible factor, Carotid body, Intermittent hypoxia, Sleep apnea

Introduction

Hypoxia is a crucial developmental signal and physiological stimulus, and also plays an important role in the pathogenesis of cancer, cardiopulmonary diseases, and other major causes of mortality (Iyer et al., 1998). In humans, intermittent hypoxia (IH) associated with recurrent episodic apnea in sleeping disorder leads to the pathologic development of cardiovascular morbidity including systemic hypertension and stroke (Fletcher, 2001). In contrast, chronic hypoxia (CH) with sustained and mild-to-moderate level of hypoxia, such as in subjects physiologically acclimatized to high-altitude, does not mirror the adverse impacts mediated by IH. The difference in the physiological and pathological consequences of CH and IH suggests distinct regulatory mechanisms responding to these two patterns of hypoxia.

Type-I (glomus) cells are the chemosensitive cells in the carotid body (CB). These cells respond to changes in arterial oxygen, carbon dioxide and acidity, and increase intracellular calcium for the vesicular secretion of neurotransmitters, including catecholamines, acetylcholine and ATP, which transduce the chemical signals into afferent nerve activity to the brain for the regulation of cardiorespiratory performance, to compensate the metabolic needs (Gonzalez et al., 1994; Iturriaga and Alcayaga, 2004; Nurse, 2005). Significant structural and functional changes occur in the CB during CH. The enhanced hypoxic sensitivity of CB in CH is due to alterations in neurotransmitter dynamics, such as upregulation of tyrosine hydroxylase (TH), and recruitment of additional neuromodulators, such as endothelin-1 (ET-1) in the glomus cell (Chen et al., 2002a,b; Wang and Bisgard, 2002; Prabhakar and Jacono, 2005). Also, morphological alterations of the CB include an enlargement of the organ with hypertrophy and hyperplasia of glomus cells, and neovascularization,

which are in part due to upregulation of the expression of vascular endothelial growth factor (VEGF) (Wang and Bisgard, 2002; Prabhakar and Jacono, 2005). These profound morphological and neurochemical changes in the CB during CH, however, are not like those of IH, in which no morphological differences have been found (Peng et al., 2003). Besides, IH augments CB sensitivity to hypoxia, but in a selective manner. It causes a novel form of plasticity in the CB, leading to long-term facilitation in the sensory discharge and this could account for the abnormally elevated sympathetic activities mediated by the chemoreflex in patients with recurrent sleep apnea (Prabhakar, 2001; Prabhakar et al., 2001; Peng et al., 2003; Rey et al., 2004).

Hypoxia inducible factor (HIF)-1 functions as a global regulator of oxygen homeostasis. HIF-1 activity is induced by hypoxia in all nucleated cell types via a novel post-translational mechanism and plays a critical role in the response of the cardiorespiratory system to hypoxia (Semenza, 2004b). Ventilatory acclimation to CH is markedly attenuated in mutant mice partially deficient in HIF-1α, which is the heterodimeric subunit of HIF-1 regulated by cellular oxygen level (Prabhakar and Jacono, 2005). Also, the mutant mice had impaired CB-mediated systemic responses to IH (Peng et al., 2006). Besides HIF-1α, two other members of the basic helix-loop-helix/PAS superfamily have also been described: HIF-2α, referred to also as endothelial PAS domain protein-1 (EPAS-1), which bears a functional resemblance to HIF-1α regarding hypoxic stabilization and binding to HIF-1α (Wiesener et al., 1998), and recently HIF-3α (Heidbreder et al., 2003). Responses to changes in oxygen pressure are primarily regulated by HIF-1α and HIF-2α (Wang et al., 1995; Iyer et al., 1998; Tian et al., 1998; Wiesener et al., 1998). Hypoxia stabilizes the transcription factor against proteasomal degradation (Maxwell et al., 1999; Cockman et al., 2000; Ohh et al., 2000; Tanimoto et al., 2000) and thereby induces the expression of spectrum of HIF-regulated genes, including VEGF, ET-1 and TH, involved in maintaining oxygen homeostasis in various tissues (Wang et al., 1995; Ema et al., 1997; Iyer et al., 1998; Tian et al., 1998) including the CB (Semenza, 2000, 2004a; Fung, 2003; Fung and Tipoe, 2003). In PC12 cells, the transcriptional expression and activity of HIF-1α protein are divergent when subjected to CH and IH (Yuan et al., 2005). The expression of HIF-1α and the HIF-regulated genes are indispensable in the transcriptional regulation of structural remodeling and functional modulation of the CB in CH (Fung, 2003; Tipoe and Fung, 2003; Roy et al., 2004). Furthermore, HIF-2α and HIF-3α are constitutively expressed in the CB (Lam et al., 2006a). Thus, the HIF-α subtypes in the CB may have diverse functions in responding to CH and IH.

The regulation and roles of HIF-α subtypes in the CB are unclear. It is important to analyze the target genes of HIF-1α and HIF-2α and comprehend their individual functions in the CB in hypoxia. Thus, the aim of the study was to characterize the temporal expression of HIF-α subtypes and the regulated gene products, including VEGF, ET-1 and TH, in the rat CB during CH and IH. We hypothesized differential expression and roles of HIF-α subtypes in the structural and functional alterations of the CB in responding to CH and IH.

Materials and methods

Chronic and intermittent hypoxia

The experimental protocol for this study was approved by the Committee on the Use of Live Animals in Teaching and Research of The University of Hong Kong. Male Sprague-Dawley rats aged 28 days (ca. 100-150 g) at the start of experiment were randomly divided into groups for the treatment of CH or IH and for normoxia (Nx). Both CH and IH rats were kept in acrylic chambers for normobaric hypoxia in the same room and had free access to water and chow. For CH, rats were placed in a 300-liter acrylic chamber, vented by room air (2 liter/min). The oxygen fraction inside the chamber was kept at 10±0.5%, 24 h per day for the CH group and was cyclic from 21 to 5±0.5% per min, 60 cycles/h, 8 h per day diurnally for the IH group. The oxygen level was continuously monitored by an oxygen analyser (Vacumed, CA, USA) which provided the servo-feedback signal for the control of solenoid values which gated the inflow of pure nitrogen. For IH, rats were placed in a 50-liter acrylic chamber with the inspired oxygen level fell to 4-5% (nadir arterial oxygen saturation ca. 70%) for about 15 sec per min, which mimicks the recurrent episodic hypoxemia in patients with obstructive sleep apnea (Fletcher, 2001). For Nx controls, aged-matched animals were kept in room air in the same housing and maintenance matching the hypoxic groups as reported previously (Tipoe and Fung, 2003). The rats were exposed to hypoxia for 3, 7, 14 and 28 days and were immediately used in experiments after being taken out of the chambers. Following deep anesthesia with halothane, rats were decapitated and the carotid bifurcation was excised rapidly.

Immunohistochemistry and double staining

Immunohistochemical staining was performed on deparaffinized formalin-fixed tissue sections of the carotid bifurcation, following antigen retrieval using 0.1 M citric acid buffer, pH 6.0, for 10 min at 98°C. After blocking of endogenous peroxidase activity with 3% hydrogen peroxide and of non-specific binding for the antiserum with 20% normal horse serum, sections were incubated with primary antibodies to the following proteins: HIF-1α (mouse monoclonal IgG antibody, 1:25 dilution, Cat # 400080, Calbiochem, CA, USA); EPAS-1 (goat polyclonal antibody, 1:200 dilution, Cat # sc-8712, Santa Cruz, CA, USA); HIF-3α (goat polyclonal

Table 1. RT-PCR primer sequences

Gene Name	RT-PCR primer (5' to 3')	
	Forward	Reverse
HIF-1α	AAGAAACCGCCTATGACGTG	CCACCTCTTTTTGCAAGCAT
HIF-2α	CCCCAGGGGATGCTATTATT	GGCGAAGAGCTTATAGATTA
HIF-3α	AGAGAACGGAGTGGTGCTGT	ATCAGCCGGAAGAGGACTTT
TH	TCGCCACAGCCCAAGGGCTTCAGAA	CCTCAAGCGCACAAAATAC

antibody, 1:250 dilution, Cat # sc-8718, Santa Cruz, CA, USA); VEGF (mouse monoclonal IgG antibody, 1:1000 dilution, SC-7269, Santa Cruz Biotechnology Inc., USA); ET-1 (mouse monoclonal IgG antibody, 1:100 dilution, Cat # CP44, Oncogene, CA, USA); TH (rabbit IgG antibody, 1:1000 dilution, Cat # AB151, Chemicon International Inc., CA, USA), in 0.05 M Tris-HCl buffer containing 2% bovine serum albumin (BSA) respectively at 4°C overnight. Detection was performed with streptavidin-biotinylated peroxidase-linked reagents (LSAB kit, Dako, CA, USA) with diaminobenzidine. For double staining, three sets of primary antibodies were used: (1) HIF-1α and TH; (2) EPAS-1 and TH; and (3) HIF-3α and TH in sections. Detection was performed simultaneously with a horseradish peroxidase-linked or an alkaline phosphatase-linked reagent (Envision® Doublestain System, Dako, CA, USA) with diaminobenzidine and Fast Red solution as the chromogens. Control sections were incubated with normal serum and stained uniformly negative.

Reverse transcription polymerase chain reaction (RT-PCR)

Four CBs were pooled for the isolation of total RNA and RT-PCR. Isolated RNA (5 μg) was subjected to first strand cDNA synthesis using random hexamer primers and Superscript II transcriptase (GIBCO, USA) in a final volume of 20 μl. After incubation at 42°C for 1 h, the reaction mixture was treated with RNase H before proceeding to PCR analysis. The final mixture (2 μl) was directly used for PCR amplification. Primer sequences are listed in Table 1. All RNA was shown to be free of DNA contamination by RT-PCR without addition of reverse transcriptase. The PCR conditions are listed in Table 2. Aliquots of the PCR products were electrophoresed on 2% agarose gels stained with ethidium bromide and photographed.

Volumetric and image analysis

The immunoreactivities of HIF-1α, HIF-2α, HIF-3α, VEGF, ET-1 and TH were measured using the Leica QWIN Imager Analyzer (Cambridge, UK) as reported previously (Tipoe and Fung, 2003). Briefly, the luminance incident light passing through each section was calibrated where the grey pixel values were set to 0

Table 2. RT-PCR conditions.

Gene Name	RT-PCR conditions		
	Denaturing	Annealing	Elongating
HIF-1α	40 cycles, 95°C, 30 s	60°C, 1 min	72°C, 2 min
HIF-2α	35 cycles, 95°C, 30 s	60°C, 1 min	72°C, 30 s
HIF-3α	35 cycles, 95°C, 30 s	60°C, 1 min	72°C, 1 min
TH	35 cycles, 94°C, 1 min	53°C, 2 min	72°C, 3 min

and 1.00. Five fields per section from one CB of each animal were measured. The percent area of positive stain for the VEGF, ET-1 and TH proteins was quantified by total area of positive brown cytoplasmic stain divided by the sum area of the reference field. For HIF-α subtypes, percentage of positive nuclei was determined by the total number of positive nuclei divided by the total number of nuclei in the reference field. A total of 20 fields for 4 CBs from four different animals at each time-point were determined. The mean value of the 20 fields was calculated to represent each time-point. Volumetric measurement was determined by obtaining corrected mean diameter (D) of the CB, then converting D to an equivalent volume of a sphere (Tipoe et al., 1992).

Statistical analysis

GraphPad Prism® software (GraphPad Software, Inc., San Diego, USA) was used to analyze the data. A non-parametric Mann-Whitney U-test was used to compare the differences between time-points. Statistical significance was set at a p-value < 0.05.

Results

The effect of CH and IH on CB size

Studies with volumetric analysis of the diameter (Fig. 1A), area (Fig. 1B) and volume (Fig. 1C) of the CB showed significant increases of the CB size in CH from 7 to 28 days, when compared with the corresponding age-matched IH and Nx groups. In contrast, the increase in CB size during IH was not significantly different from the Nx group except for the 28-day group. The CB volume in CH increased by ca. 2 to 6 times that of the Nx control, whereas the increase was less than 2.5-fold

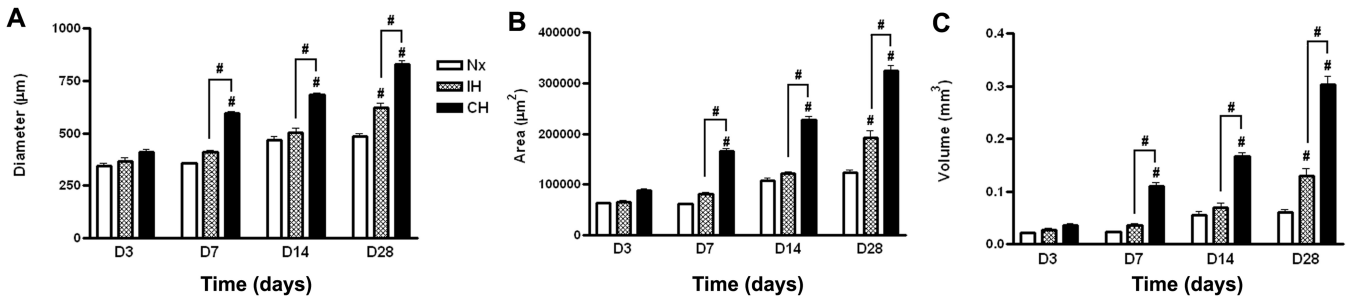**Fig. 1.** Volumetric measurement of the CB of rats in CH, IH and Nx for 3 (D3), 7 (D7), 14 (D14) and 28 days (D28). Measurements are diameter **(A)**, area **(B)** and volume **(C)**. Data are expressed as means + SEM. (n = 10 for each group). #$p < 0.001$ vs. corresponding Nx or as indicated by brackets.
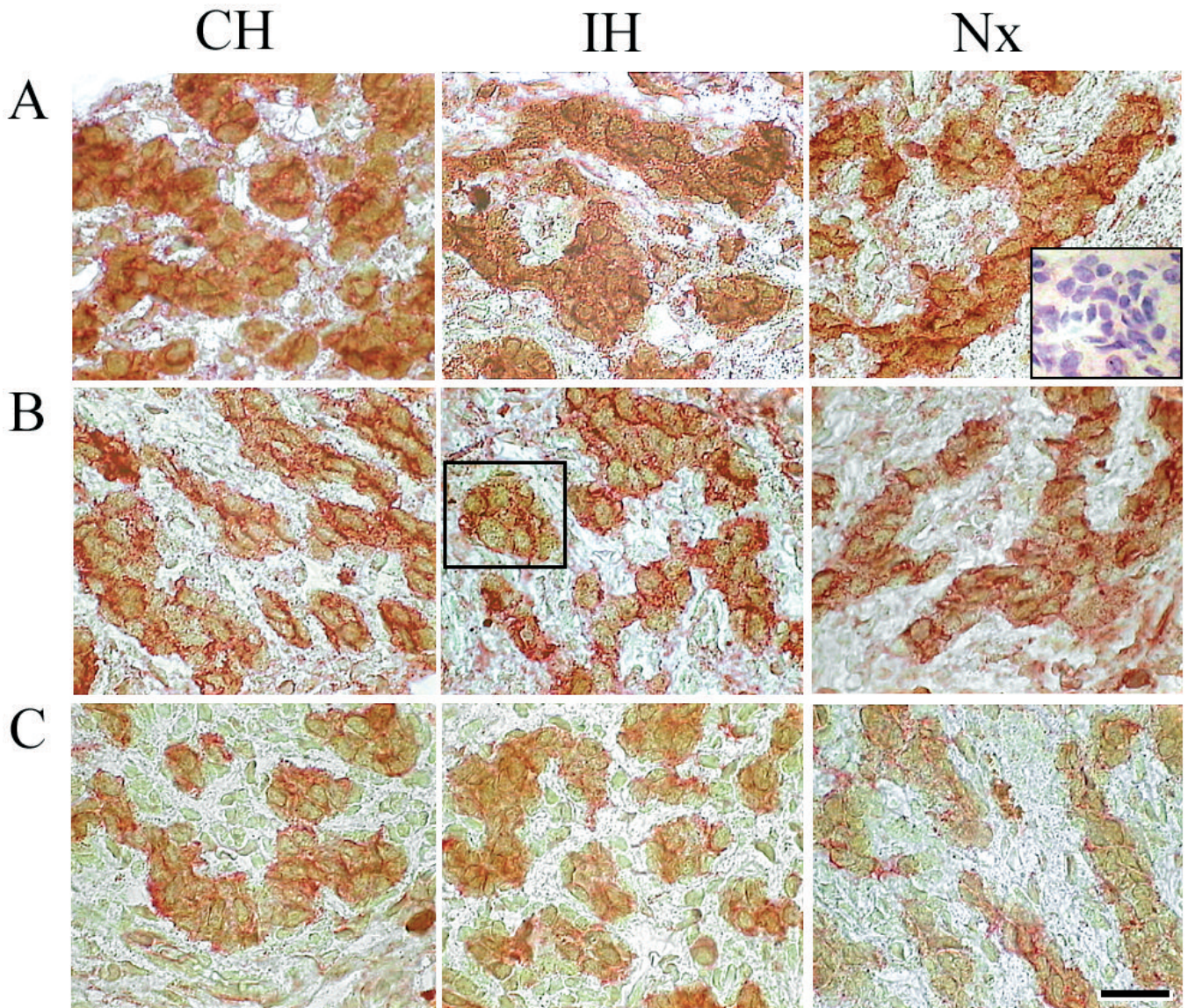

Fig. 2. Immunohistochemical co-localization of HIF-α subtypes and TH in the CB of CH, IH and Nx rats with 7-day treatment. Immunoreactivities for HIF-α subtypes were localized in cell clusters of glomus cells (black squares) with TH positive staining, as demonstrated by double immunostaining. Distinct immunoreactivity for HIF-1α **(A)**, HIF-2α **(B)** and HIF-3α **(C)** was mainly found in nuclei of glomic cells (brown stain) and the immunoreactivity for TH was localized in the cytoplasm of the glomus cells (red stain) in clusters in the CB. No immunostaining was observed in CB glomic cells with the replacement of primary antibody with the corresponding normal IgG in the HIF-α subtypes counterstained with haematoxylin (black square on upper right). Bar: 20 μm.

with IH for 28 days (Fig. 1C). These results demonstrated differences in volumetric remodeling of the CB during CH and IH, suggesting differential CB responses to CH and IH.

Expression of HIFs in the CB in CH and IH

To determine if the morphological difference of the CB in CH and IH may be due to differential HIF expression in the CB tissue, immunohistochemical double-staining revealed that the immunoreactivity (IR) of HIF-α subtypes was found in the nuclei of clusters of cells with TH-positive staining (Fig. 2), indicating a co-localization of the HIF-α subtypes expression in the CB glomic tissue. There was no positive staining in the CB sections with the replacement of the primary antibody with the corresponding normal IgG in any of the HIF-α subtypes (Fig. 2; upper right black square). Image analysis measuring the % nuclei with HIF-1α expression showed a significant increase in the CH, but not in the IH group, when compared with that of the Nx. The proportional amount of cells expressing HIF-1α was *ca.* 45 to 65% in the CH group, but was much less (ca. 15 to 35%) in the IH group, which was comparable to that of the Nx group (Fig. 3A).

The proportional amount of CB cells expressing HIF-2α and HIF-3α were increased at day 3 in both the CH and IH groups, and were sustained at a higher level than the Nx throughout the time course (Fig. 4A, 5A). To test whether the alterations in HIF-α subtypes expression were mediated by transcriptional regulation, RT-PCR studies were performed on the CB from 7-day CH and IH rats. Results showed that mRNA expressions of the HIF-α transcripts were upregulated in the CB of both the CH and IH rats when compared with the Nx control. In addition, the mRNA levels of the HIF-α transcripts were higher in the IH than those of the CH group, suggesting a differential control at the transcriptional level (Figs. 3B, 4B, 5B).

Expression of VEGF and ET-1 in the CB of CH and IH rats

To examine whether differences in the HIF-α subtypes expression could result in dissimilar expression of the HIF-regulated gene products involved in the CB remodeling during CH and IH, we analyzed the profiles of VEGF and ET-1 expression in the CB of CH and IH rats. As reported previously (Chen et al., 2002b; Tipoe and Fung, 2003), the immunoreactivity of VEGF and ET-1 was mainly localized in the cytoplasm of the glomic clusters of the CB. Image analysis measuring the % CB area with positive immunostaining of VEGF showed significant increases in the 7- and 28-day CH groups when compared with the age-matched Nx control (Fig. 6A). Conversely, no significant difference was observed in the IH throughout the time-course (Fig. 6A). The proportional amount of CB area expressing ET-1 was markedly increased in day 3 of both CH and IH

groups, although the elevation was less in the IH than that of the CH group (Fig. 6B). The elevation gradually reduced to levels above the Nx control by day 7 and the IH group completely recovered to the Nx level by 28 days (Fig. 6B). Results show dissimilar profiles of VEGF and ET-1 expression in the CB tissue in responding to CH and IH.
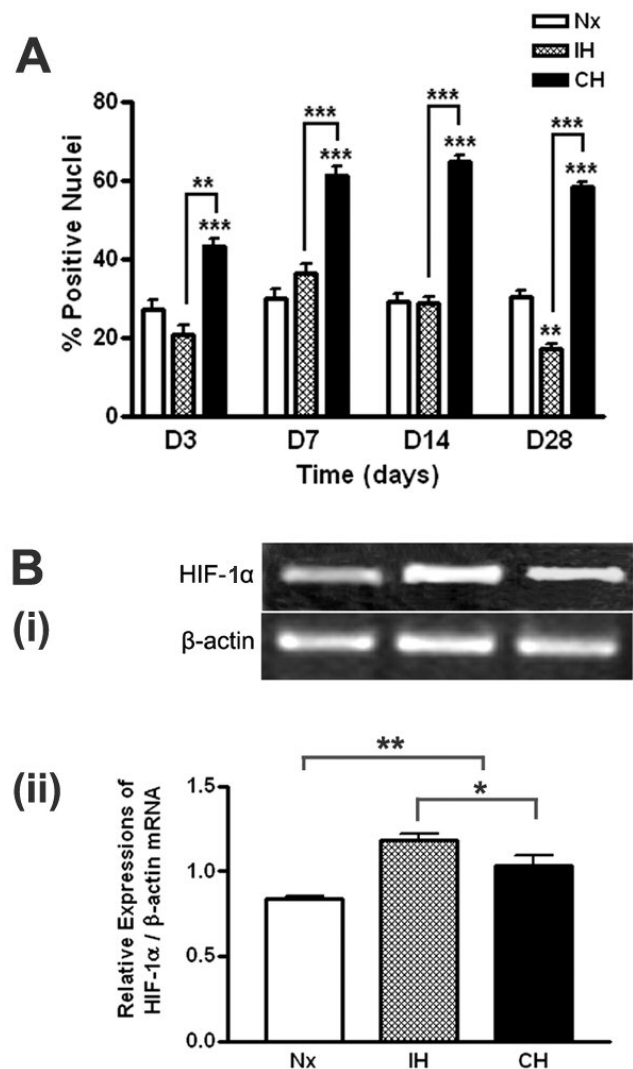

Fig. 3.A. Protein expressions of HIF-1α in the CB of rats in CH, IH and Nx for 3 (D3), 7 (D7), 14 (D14) and 28 days (D28). Data are presented in % positive nuclei with positive staining of HIF-1α immunoreactivity. Data are expressed as means + SEM. (n=5 for each group). **p<0.005; ***p<0.0005 vs. corresponding Nx or as indicated by brackets. **B.** RT-PCR analysis of the mRNA expression of HIF-1α. **i.** Gel image of the RT-PCR products of the HIF-1α in 7-day Nx, CH and IH rat CB. The expected size of PCR products from HIF-1α is 301 bp and ß–actin is 436 bp. **ii.** The relative expressions of HIF-1α/ß-actin mRNA (% of control). Columns show means + SEM. (n=5 for each group). *p<0.05; **p<0.005; as indicated by brackets.

Expression of TH in the CB of CH and IH rats

Functional modulation of the CB in hypoxia involves changes in the TH (the rate-limiting enzyme of catecholamine synthesis) expression, which is regulated by HIF. The profile of TH expression was examined in the CB of CH and IH rats. As shown in Fig. 2, the immunoreactivity of TH was mainly localized in the cytoplasm of the glomic clusters of the CB. Image analysis measuring the % CB area with positive immunostaining of TH showed significant increases in both CH and IH groups when compared with the Nx control. The proportional CB area expressing TH

remained at elevated levels throughout the time course in hypoxia, and the CH group had a higher expression than that of the IH group except at day 7 (Fig. 7A). In addition, mRNA expressions of the TH transcript in the CB of both the CH and IH rats at day 7 were significantly increased by ca. 2-fold of the Nx control (Fig. 7B).

Discussion

The major finding of this study is that the structural changes in the CB in responding to IH and CH are mediated by HIF pathways regulated at transcriptional and post-translational levels. However, different HIF-α
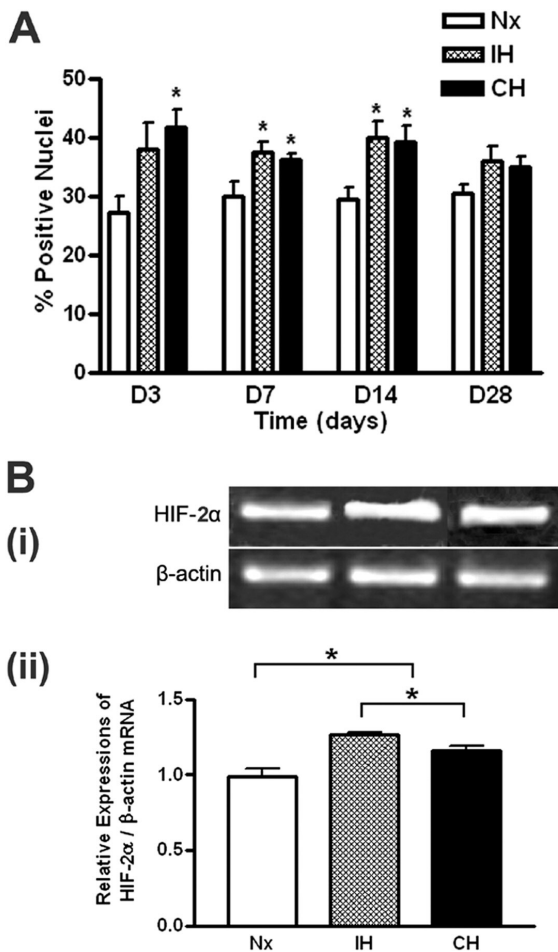

Fig. 4.A. Protein expressions of HIF-2α in the CB of rats in CH, IH and Nx for 3 (D3), 7 (D7), 14 (D14) and 28 days (D28). Data are presented in % positive nuclei with positive staining of HIF-2α immunoreactivity. Data are expressed as means + SEM. (n=5 for each group). *p<0.05 vs. corresponding Nx. **B.** RT-PCR analysis of the mRNA expression of HIF-2α. **i.** Gel image of the RT-PCR products of the HIF-2α in 7-day Nx, CH and IH rat CB. The expected size of PCR products from HIF-2α is 298 bp and β–actin is 436 bp. **ii.** The relative expressions of HIF-2α/β-actin mRNA (% of control). Columns show means + SEM. (n=5 for each group). *p<0.05; as indicated by brackets.
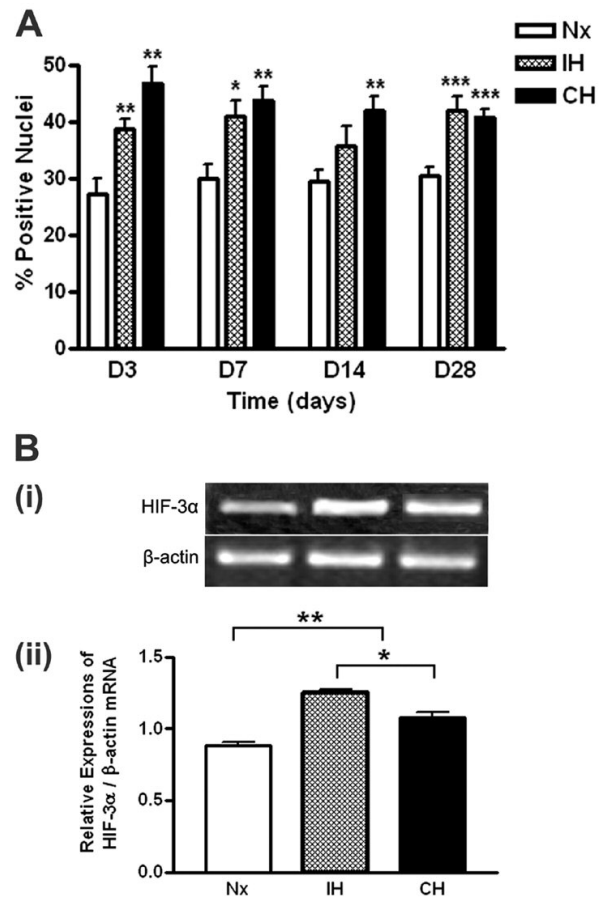
Fig. 5.A. Protein expressions of HIF-3α in the CB of rats in CH, IH and Nx for 3 (D3), 7 (D7), 14 (D14) and 28 days (D28). Data are presented in % positive nuclei with positive staining of HIF-3α immunoreactivity. Data are expressed as means + SEM. (n=5 for each group). *p<0.05; **p<0.005; ***p<0.0005 vs. corresponding Nx. **B.** RT-PCR analysis of the mRNA expression of HIF-3α. **i.** Gel image of the RT-PCR products of the HIF-3α in 7-day Nx, CH and IH rat CB. The expected size of PCR products from HIF-3α is 301 bp and α–actin is 436 bp. **ii.** The relative expressions of HIF-3α/β-actin mRNA (% of control). Columns show means + SEM. (n=5 for each group). *p<0.05; **p<0.005; as indicated by brackets.

subtypes play separate roles in the responses to CH and IH, leading to differential profiles of the expression of HIF-target gene products that cause the differences in the structural remodeling and functional modulation of the CB in CH and IH. Chemoreflex activated by the CB plays a central role in cardiorespiratory responses to hypoxia. The CB in CH develops structural changes, including hypertrophy of the organ with increased vascularization and number of glomus cells (Dhillon et al., 1984; Lahiri et al., 2000; Wang and Bisgard, 2002). However, chronic IH selectively augments CB sensitivity to hypoxia and causes long-lasting activation of sensory discharge (Prabhakar, 2000); yet no significant changes in the CB volume and number of glomus cells were reported in rats with 10 days of IH (Prabhakar, 2001; Peng et al., 2003). Our data inevitably supports these observations, showing the hypertrophied

response to CH, in which CB volume was found to be increased significantly from 7 days of CH, and the CB was enlarged by 6-fold with 28 days of CH. In contrast, CB volume in the IH group was not different from the Nx control from day 3, 7 and 14, and the increase in the CB volume was significantly less in the IH (ca. 2.5 fold) than that of the CH group at day 28. Hence, the time course for the hypertrophied response to CH and IH is different, suggesting differential mechanisms underlying
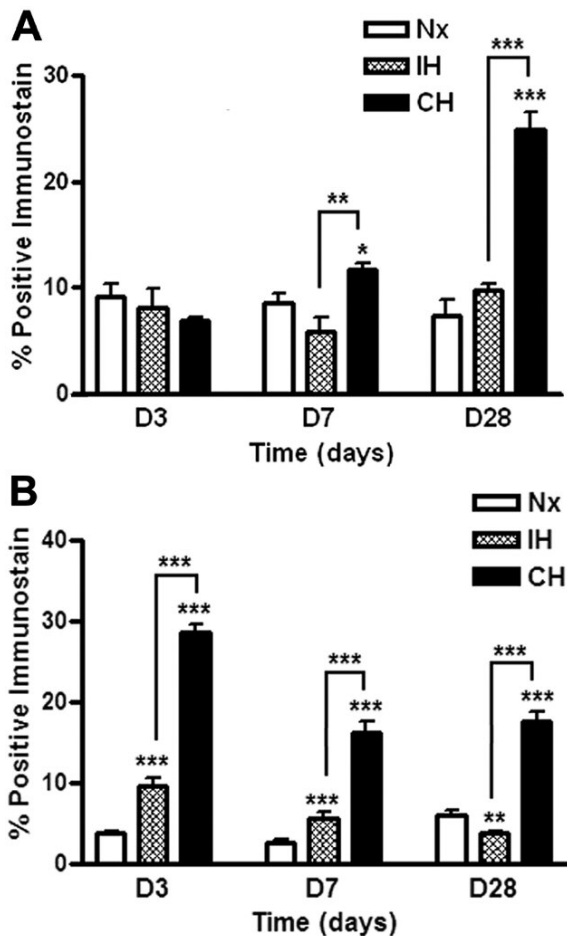

Fig. 6. Protein expressions of VEGF **(A)** and ET-1 **(B)** in the CB of rats in CH, IH and Nx for 3 (D3), 7 (D7), 14 (D14) and 28 days (D28). Data are presented in % area with positive staining of VEGF or ET-1 immunoreactivity. Data are expressed as means + SEM. (n=5 for each group). *p<0.05; **p<0.005; ***p<0.0005 vs. corresponding Nx or as indicated by brackets.
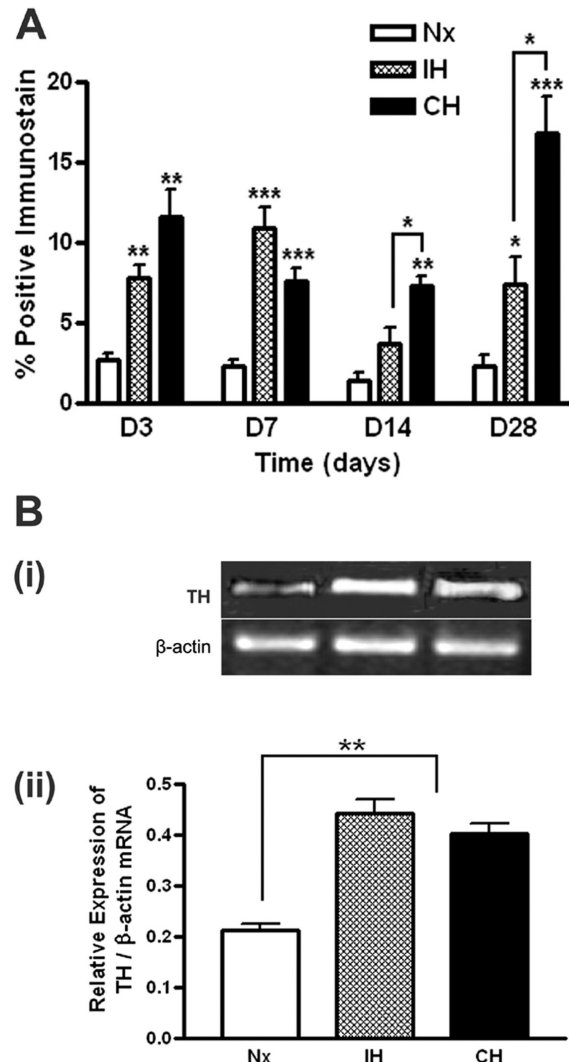

Fig. 7.A. Protein expressions of TH in the CB of rats in CH, IH and Nx for 3 (D3), 7 (D7), 14 (D14) and 28 days (D28). Data are presented in % area with positive staining of TH immunoreactivity. Data are expressed as means + SEM. (n=5 for each group). *p<0.05; **p<0.005; ***p<0.0005 vs. corresponding Nx or as indicated by brackets. **B.** RT-PCR analysis of the mRNA expression of TH. **i.** Gel image of the RT-PCR products of the TH in 7-day Nx, CH and IH rat CB. The expected size of PCR products from TH is 368 bp and α–actin is 436 bp. **ii.** The relative expressions of TH/α-actin mRNA (% of control). Columns show means + SEM. (n=5 for each group). **p<0.005; as indicated by brackets.

the CB responses to CH and IH.

HIF-1α is expressed in most cell types, whereas HIF-2α shows a more restricted pattern of expression that includes the developing lung, vasculature, and catecholamine-producing cells. In the CB, HIF-1 has been shown to play a key role in mediating the structural and physiological responses to CH (Kline et al., 2002; Fung, 2003; Tipoe and Fung, 2003; Semenza, 2004a; Di Giulio et al., 2005). In addition, HIF-2α and HIF-3α are correspondingly expressed in the glomus cells in the CB, the expression of which are also activated by CH (Lam et al., 2006a). Here we have demonstrated different temporal profiles of the expression of the HIF-α subtypes in the CB during CH and IH. Results showed an increase in proportional amount of CB cells expressing HIF-1α, HIF-2α and HIF-3α during CH, suggesting that HIF-2α and HIF-3α play complementary roles to the HIF-1α in the CB response to hypoxia. This is also supported by the fact that there is an increase in the HIF-2α and HIF-3α but not HIF-1α expression in the IH, except on day 7. Apparently, the CB structural remodeling could depend on the HIF-1α expression, because the significant increases in the CB volume occur in CH but not IH. In addition to HIF-1α, the expression of HIF-2α and HIF-3α could play roles in the functional modulation of the CB during hypoxia. This is particularly implicated in the enhanced CB chemosensitivity during IH. Nevertheless, mRNA transcripts of all HIF-α subtypes were upregulated by CH and IH, suggesting that all HIF-α genes are responsive to hypoxia mediated by regulatory mechanisms at the transcriptional level. The subtle but statistically significant difference in the level of HIF-α mRNA transcripts between the CH and IH groups may reflect distinct regulatory pathways induced by CH and IH. In this context, the production of ROS in the CB during IH is known to be a critical factor in altering the CB function and HIF-1 activation in IH (Prabhakar, 2001; Prabhakar et al., 2001; Peng et al., 2006). Furthermore, it is well defined that post-translational modification plays an essential role in determining the protein level of HIF-1α, which depends on the cellular oxygen level (Lahiri et al., 2006). The oxygen tension in the CB tissue may rapidly recover to normoxic level following the hypoxic episodes during IH, which could permit the modification for keeping the HIF-1α protein at a low level. Alternatively, differential pathways induced by CH and IH for the post-translational control of HIF-1α could account for the discrepancy.

When hypoxia persists for prolonged period of time, the CB response involves changes in gene expression regulated by HIF. In fact, partial HIF-1α deficiency specifically impairs CB ability to sense O_2 and respond to hypoxia, indicating that HIF-1α plays an important role in the responses of the CB to both CH and IH (Kline et al., 2002; Peng et al., 2006). Also, the increase in the HIF-2α and HIF-3α expression in the CB during CH and IH suggests that the HIF-α subtypes play differential roles in the CB. Target genes that are transactivated by

HIFs containing HIF-1α, HIF-2α or HIF-3α could encode different proteins that play unique roles in the CB responses to CH and IH. Our results showed that the protein expression of VEGF, a common target gene regulated by HIF-1α and HIF-2α, was significantly increased in the CB of the CH but not the IH group. VEGF induces angiogenesis and it also increases permeabilization of the vasculature in tissues and organs (Ferrara et al., 1992; Neufeld et al., 1999). CB hypertrophy is closely associated with VEGF expression and vasculature remodeling (Wang and Bisgard, 2002; Fung, 2003; Prabhakar and Jacono, 2005; Tipoe et al., 2006). Thus, different profiles of the protein expression of VEGF in the CB could account for the morphological differences of the organ in CH and IH. In fact, the temporal profile of VEGF expression was in parallel with the changes in CB volume. Also, a lack of an increase in HIF-1α protein in the IH group could result in the absence of VEGF response to IH, leading to the insignificant increase in the CB volume.

In addition to VEGF expression, HIF-1 regulates the expression of ET-1 and the enhanced ET-1 expression plays a role in CB hypertrophy in CH. It has been shown that ET-1 increases intracellular calcium in the glomus cell and mediates the proliferative response of the cells during CH (Chen et al., 2002a,b). Our results accentuate a robust ET-1 expression in CH, but it was less in the IH. This may account for the insignificant enlargement of CB in IH, because mitogenic effect on the glomus cell induced by ET-1 in IH is less than that of the CH (Lam et al., 2006b). Thus, the VEGF and ET-1 expression regulated by HIF-1 is insignificant in the IH compared with the CH response, and this could be responsible, at least in part, for the difference in volumetric remodeling of the CB in CH and IH.

The rate-limiting enzyme in the biosynthesis of catecholamines, TH, is induced by hypoxia at the level of gene expression. Through an inhibition of the von Hippel-Lindau tumor suppressor protein (pVHL) and HIF-α ubiquintination, hypoxia induces TH gene transcription and stabilizes TH mRNA in PC12 cells (Czyzyk-Krzeska et al., 1994). Physiological regulation of the TH gene by hypoxia occurs in glomus cells of the CB (Czyzyk-Krzeska et al., 1992). Our results demonstrated that TH mRNA was upregulated in both CH and IH, which is consistent with the finding that TH mRNA expression in the CB is stimulated by hypoxia (Czyzyk-Krzeska et al., 1992). It has been shown that HIF-1α and HIF-2α have similar efficacies on the TH promoter, and both HIFs are expressed at comparable levels in the PC12 cells (Schnell et al., 2003). Although both HIF-1α and -2α play an important role in CB responses in hypoxia, we showed an increase in the expression of HIF-2α, but not HIF-1α protein in the CB during IH, suggesting a major role played by HIF-2α in TH expression during hypoxia. In this context, HIF-2α is the primary HIF expressed in the catecholaminergic cells in the CB during development (Tian et al., 1998). In mice with partial HIF-2α deficiency, hypoxia-induced

expression of norepinephrine and ET-1 was dramatically impaired, and the mice did not develop pulmonary hypertension after 4 weeks of exposure to 10% O_2 (Semenza, 2004a). Thus, HIF-2α in the CB may play a significant role in the regulation of TH expression for the increase in synthesizing catechoamines. Furthermore, the increase in HIF-2α expression could be important for the elevation of ET-1 expression in the CB during hypoxia, particularly in the early time-course of IH. In addition to the mitogenic effect of ET-1 on the glomus cell, the local increase of ET-1 in the CB can contribute to the enhanced chemosensory responses induced by IH predominantly through a vasomotor mechanism (Rey et al., 2006). Thus, together with the increased TH expression, the ET-1 expression regulated by HIF-2α in hypoxia could be functionally significant in the enhancement of the CB chemosensitivity in the responses to IH and to CH as well (Chen et al., 2002a,b; Lam et al., 2006b). Moreover, hypoxia also activates HIF-3α expression (Heidbreder et al., 2003). Our results inevitably support this, in which both the transcriptional and post-transcriptional levels of HIF-3α were upregulated in CB in CH and IH. However, there is a paucity of information on the target genes regulated by HIF-3α and its functional role in the CB remains to be further investigated. Nevertheless, it is speculated that HIF-3α upregulation could be complementary to the expression and functions of HIF-1α and -2α under hypoxic conditions.

In conclusion, the CB glomic tissue responded differently to CH and IH, which had distinct profiles of the expression of HIF-α subtypes. The increased levels of the HIF-2α and HIF-3α protein and mRNA may be complementary to the role of HIF-1α in modulating the chemoreceptor function in CH and IH. The differences in HIF-1α and HIF-2α targets may contribute to the differential effects of these factors on the CB in IH. Taken together, differential regulation of HIF-α expression and HIF-regulated gene products could account for the CB morphological and neurochemical discrepancies in CH and IH.

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