Expression of heme oxygenase in the oxygen-sensing regions of the rostral ventrolateral medulla

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Mazza, Emilio, Smita Thakkar-Varia, Carol A. Tozzi, and Judith A. Neubauer. Expression of heme oxygenase in the oxygen-sensing regions of the rostral ventrolateral medulla. J Appl Physiol 91: 379–385, 2001.—Recently, unique regions in the rostral ventrolateral medulla (RVLM) have been found to be oxygen sensitive. However, the mechanism of sensing oxygen in these RVLM regions is unknown. Because heme oxygenase (HO) has been shown to be involved in the hypoxic responses of the carotid body and pulmonary artery, the aim of this study was to determine whether HO is present in the RVLM and whether expression of HO is altered by chronic hypoxia. Adult rats were exposed to hypoxia (10% O2) or normoxia (21% O2) for 10 days, and the mRNA for HO-1 and HO-2 was examined in the RVLM by using RT-PCR. Expression of HO-2 mRNA was seen in the RVLM of both control and hypoxic samples, whereas expression of HO-1 mRNA was only seen in the RVLM of hypoxic samples. HO-2 was immunoocytochemically localized in brain sections (40 μm) to the C1 region and pre-Bötzinger complex of the RVLM. Together, these results indicate that HO-2 is present in the RVLM under control conditions and that HO-1 is induced in the RVLM during chronic hypoxia, consistent with a potential role for HO in the oxygen-sensing function of these cardiopulmonary RVLM regions.

CARDIORESPIRATORY NEURONS within the rostral ventrolateral medulla (RVLM) have been shown to be excited by hypoxia both in vivo (36, 39) and in vitro (23, 40). Sun et al. (39) demonstrated that microinjection of NaCN in the C1 region of the RVLM produces an increase in tonic sympathetic output and an increase in arterial pressure. In addition, Solomon et al. (36) have shown that local microinjection of NaCN into the pre-Bötzinger complex, the hypothesized locus of respiratory rhythmogenesis, produces an augmentation in phrenic nerve output. The hypoxic sensitivity of these regions appears to be due to a direct oxygen sensitivity of neurons because neurons cultured from these areas of the RVLM containing both the C1 region and pre-Bötzinger complex exhibit an intrinsic excitatory response to hypoxia (15). However, the mechanism(s) underlying this chemosensitive process is unknown.

In other oxygen-sensing systems, such as the carotid body and the pulmonary artery, heme oxygenase (HO) has been found to be involved in the adaptive responses to hypoxia (4, 30, 47). HO is an oxygen-dependent enzyme catalyzing the reaction that converts heme into biliverdin and carbon monoxide, an important second messenger within the central nervous system (13). Three forms of HO have been described to date (14, 16). HO-1, an inducible isoform of the enzyme, has been shown to increase with hypoxia in the pulmonary artery and aorta and after transient ischemia within the brain (7, 22, 24, 42). HO-2 is a constitutive isoform of the enzyme whose expression is not affected by these same stimuli (7, 14). A recently described form, HO-3, closely resembles HO-2 but is not well characterized as far as stimuli that affect its expression (16). HO-1 and HO-2 expression have been shown in several areas of the brain (6), but their presence in the RVLM has not been described.

If HO is to be considered a potential candidate involved in the acute and/or adaptive responses of the cardiopulmonary regions of the rostral medulla, its presence in these regions must first be demonstrated. Thus the specific aims of this study were to determine 1) whether the constitutive form of the enzyme, HO-2, is expressed in the RVLM of adult rats during control normoxic conditions; and 2) whether the inducible form of the enzyme, HO-1, is expressed in the RVLM in rats exposed to 10 days of chronic hypoxia.

METHODS

Hypoxic exposure of adult rats. Six-week-old Sprague-Dawley rats (200–250 g) were exposed to hypoxia (10% O2-90% N2 at ambient pressure; n = 8) while corresponding age-matched controls were maintained in room air (21% O2; n = 8) in a Plexiglas chamber for 10 days. The Plexiglas chamber was monitored via a computer for percent oxygen and carbon dioxide and contained soda lime to maintain carbon dioxide at atmospheric levels. Plexiglas chamber temperature was maintained at 37°C during the entire exposure period.
period. Animals were fed ad libitum with standard rat chow and water.

**RT-PCR.** For RT-PCR experiments, animals were anesthetized with pentobarbital sodium (30 mg/kg) and the brain, aorta, pulmonary artery, and lung were quickly dissected. Because HO-2 is present in the lungs and pulmonary and systemic arteries and HO-1 is inducible under hypoxic conditions (3, 17, 20, 43, 47), samples of the lung, aorta, and pulmonary artery were used as positive controls. The brain was dissected to remove the cerebral hemispheres, cerebellum, and brain stem. Cerebral hemisphere samples consisted of the entire cortex, including the subcortical nuclei. The brain stem was further dissected to remove the RVLM. The pulmonary artery trunk, the entire left extrapulmonary artery, and proximal 3 mm of the right pulmonary artery were excised en bloc. The aorta sample included the proximal ascending aorta, aortic arch, and part of the descending aorta. Lung samples were derived from peripheral lung tissue and contained muscular pulmonary arteries. All studies were done twice on individual pooled tissues from their respective oxygen-exposure group.

Total RNA (50 µg), from control and hypoxic tissues, was extracted by the guanidine isothiocyanate method and employed to synthesize single-stranded cDNA by RT-PCR by adding 750 U Moloney murine leukemia virus reverse transcriptase and 50–100 pmol of oligo(dT)12–18. An aliquot of the single-stranded cDNA was primed with the two oligonucleotide primers 5'-GAA TTC AGC ATG CCC CAG GAT TTG-3' and 5'-TCT AGA CTA GCT GGA TGT TGA GCA GGA-3' complementary to the rat HO-1 cDNA to amplify a 615-bp fragment and 5'-GAA TTC GGG ACC AAG GAA GCA CAT-3' and 5'-TCT AGA CTA GCT GGA TGT TGA GCA GGA-3' complementary to the rat HO-2 cDNA to amplify an 828-bp fragment (20). PCR fragments generated using these primer pairs had been cloned and sequenced to confirm their identity (20). Amplification was performed in a 100-µl reaction mixture (50 mM NaCl, 10 mM KCl, 10 mM Tris-HCl, 1.5 mM MgCl2, 3 mM dithiothreitol, gelatin, and 200 µM each of nucleotide triphosphates, pH 8.8) containing 2.5 U of Taq polymerase. The reaction was carried out on a DNA thermal cycler (model 480, Perkin-Elmer Cetus Instruments) for 35 cycles as follows: denaturation at 94°C, 1.5 min; annealing at 54°C, 1 min; extension at 75°C, 1.5 min; and final extension, 10 min. The RT-PCR products were electrophoresed on an agarose gel and blotted onto nitrocellulose, and Southern blot analysis was performed.

**Immunocytochemistry and tissue sections.** Brain sections were prepared for immunocytochemical localization of HO in the following manner. Adult rats were anesthetized with pentobarbital sodium (30 mg/kg) and transcardially perfused with heparinized saline followed by 4% paraformaldehyde (in PBS, pH 7.4; Sigma Chemical) by using gravity flow for 1 h. The entire brain, including cerebellum and brain stem, was carefully removed and placed in 4% paraformaldehyde overnight at 5°C. The tissue was cryoprotected in 4% paraformaldehyde containing 30% sucrose solution for 72 h at 5°C. The tissue was then thoroughly frozen on dry ice, coated in a goat anti-rabbit secondary antibody labeled with fluorescein isothiocyanate (1:100; Sigma Chemical) diluted in PBS containing 0.01% Triton X-100, 1% goat serum, and 0.1% sodium azide for 2 h at room temperature. The sections were then washed in distilled water and mounted on gelatin-coated glass slides (Fisher) with Gelmount (Biomeda). Control sections for background staining were processed as above except sections were incubated in PBS lacking primary antibody and containing 0.3% Triton X-100 and 10% goat serum for 4 days at 5°C. Sections were visualized for immunoreactivity and photographs taken by using an upright microscope with fluorescent optics (Optiphot-Nikon). Serial sections not processed for HO-2 were directly mounted on gelatin-coated glass slides after cryosectioning and stained with neutral red to visualize cell bodies for anatomic localization of nuclei. Cell nuclei were identified according to stereotaxic anatomy as described by Paxinos and Watson (25).

**RESULTS**

**Expression of HO mRNA in the RVLM.** The expression of the mRNA for HO-2 was examined in both control and hypoxic tissues obtained from eight adult male rats (Fig. 1). Samples from the pulmonary artery, aorta, and lung served as positive controls because these tissues have been shown in previous studies to express HO-2 mRNA (17, 43, 47). Consistent with these previous reports, Fig. 1 demonstrates that an 828-bp fragment corresponding to HO-2 was seen in the pulmonary artery, aorta, and lung derived from both control (lane C) and hypoxic (lane H) rats. Expression of HO-2 was also observed in the RVLM from both control and hypoxic animals (Fig. 1). Thus the RVLM constitutively expresses HO-2 under both normoxic and chronically hypoxic conditions.

The expression of HO-1 mRNA was also examined in both control and hypoxic tissue samples of the pulmonary artery (PA), aorta, rostral ventrolateral medulla (RVLM), and lung after exposure to either 21% O2 (C) or 10% O2 (H). An 828-bp heme oxygenase-2-specific fragment was detected in both control (lane C) and hypoxic (lane H) tissues. M, molecular weight markers.

![Fig. 1. RT-PCR amplification of heme oxygenase-2 in pulmonary artery (PA), aorta, rostral ventrolateral medulla (RVLM), and lung after exposure to either 21% O2 (C) or 10% O2 (H). An 828-bp heme oxygenase-2-specific fragment was detected in both control (lane C) and hypoxic (lane H) tissues. M, molecular weight markers.](https://example.com/figure1.png)
RVLM, pulmonary artery, aorta, cerebral hemispheres, and cerebellum (Fig. 2). Expression of a 615-bp specific HO-1 fragment was not detected in tissue samples from control rats (lane C) but was induced by hypoxia (lane H) in the pulmonary artery, aorta, and RVLM. HO-1 mRNA was not detected in the cerebellum or cerebral hemispheres obtained from either control (lane C) or hypoxic (lane H) rats. HO-1 has been shown previously to be induced by hypoxia in the pulmonary artery and aorta (20, 43). Within the brain, the hypoxic induction of HO-1 mRNA expression in the brain was localized to the RVLM. Taken together, the constitutive expression of HO-2 in the RVLM, as well as the induced expression of HO-1 in the RVLM during chronic hypoxia, suggests that this enzyme may have an important function during hypoxic conditions.

Immunocytochemical localization of HO-2 in the RVLM. Immunocytochemical techniques were utilized to determine whether expression of HO-2 within the RVLM was localized to the sympathoexcitatory C1 region and respiratory pre-Bo¨tzinger complex. In addition, the expression of HO-2 was determined in regions of the brain that have been shown previously to express HO-2, such as the cerebellum, trapezoid nucleus, mesencephalic trigeminal nucleus, and facial nucleus (6). As shown in Fig. 3, immunoreactive staining for HO-2 was found in cells within the cerebellum, trapezoid nucleus, mesencephalic trigeminal nucleus, facial nucleus, and hypoglossal nucleus. Within the RVLM, HO-2 was found to be localized in cell bodies in an area containing the C1 region and pre-Bo¨tzinger complex (Fig. 4, A and B). In more caudal extents of the RVLM, the lateral reticular nucleus was also immunoreactive for HO-2 (Fig. 4, D and E). Sections of the RVLM processed without the primary antibody for HO-2 did not show any staining (Fig. 4, C and F).

DISCUSSION

The results of this study demonstrate, for the first time, expression of HO within cardiorespiratory regions of the RVLM. The expression of the mRNA of the constitutive form of the enzyme, HO-2, was maintained under normoxic and hypoxic conditions, whereas expression of the inducible form, HO-1, was only seen after exposure to hypoxia. Immunocytochemistry showed that HO-2 localized to discrete regions of the RVLM, specifically the sympathoexcitatory C1 region and respiratory pre-Bo¨tzinger complex. The functional relevance of HO in these cardiorespiratory regions was not explored in this study. However, HO regulates the production of carbon monoxide, an important chemical messenger in the brain, which could modulate the hypoxic responses of these medullary regions. Expression of HO-2 is not unique to the RVLM. With the use of in situ hybridization and immunocytochemistry, HO-2 has been found by others to be localized in a number of brain regions (6, 44). Consistent with these previous reports, we found a distribution of HO-2 within subpopulations of neurons in the central nervous system, including the cerebellum, trigeminal, trapezoid, and facial nuclei. The novel observation in this study, however, was the localization of HO in the RVLM. Within the RVLM, the mRNA for HO-2 was constitutively expressed under both control and hypoxic conditions. This constitutive pattern of expression was similar to the pattern of expression of HO-2 mRNA in the lung, pulmonary artery, and aorta that has been shown in previous studies (17, 20, 43, 47) and implies that HO-2 probably has a tonic influence on cell function. In contrast, expression of HO-1 in the brain was only seen during hypoxia and then only in the RVLM and not in the cerebellum or cerebral hemispheres, possibly implicating HO-1 in the adaptive response to chronic hypoxia. Although HO-1 expression can be induced by heat shock and focal ischemia in several regions of the brain (6, 22, 42), the observation that the chronic hypoxia used in this study did not produce a global increase in HO-1 within the brain may...
suggest that hypoxia is a specific stimulus for the induction of HO-1 in the RVLM. An alternative explanation is that, although the level of hypoxia used in this study (10% O2 for 10 days) was sufficient to stimulate transcription of the HO-1 gene in the RVLM, it may have been insufficient to stimulate expression of HO-1 in the cerebellum or cerebral hemispheres. Thus there could be a difference in the oxygen sensitivity with a lower threshold for induction in the RVLM than in other brain regions. Further studies examining different levels of hypoxia on the regional expression of HO-1 in the brain would be needed to address this question. Nevertheless, a greater sensitivity for induction of HO-1 in the RVLM may be important for the cardiorespiratory adaptations of this region during chronic hypoxia.

The expression of HO within the C1 region and pre-Bötzinger complex of the RVLM is presumably related to some function of these regions. One function of interest is the recent observation that these regions can be excited by acute localized hypoxia in vivo, resulting in an augmentation of sympathetic and respiratory output, respectively (36, 39, 41). Studies in vitro have confirmed this observation by demonstrating that a subpopulation of neurons cultured from the RVLM of neonatal rats containing the pre-Bötzinger complex and C1 region are excited by acute hypoxia (15). If HO is important for oxygen sensing in these neurons, a potential implication of the present finding is that this enzyme may be a potential marker for the hypoxia-excited neurons in these cardiorespiratory regions.

The functional significance for the presence of HO in the RVLM in both its constitutive and inducible isoforms may well be linked to the oxygen-sensing properties of this region. Oxygen sensitivity of these medullary cardiorespiratory regions could utilize similar mechanisms as other oxygen-sensitive structures. With regard to the one such organ, the carotid body, several hypotheses have been proposed to explain the oxygen-sensing mechanism, but one theory has consistently implicated the involvement of a heme-type oxygen-sensor protein in the oxygen-sensing process. This heme protein hypothesis proposes that changes in oxygen levels are sensed by a heme protein, which then, either directly or indirectly, modulates the conductance of ion channels altering the excitability of the cell (2, 29). Several heme-containing proteins have been proposed as potential candidate oxygen sensors in the carotid body, including mitochondrial cytochromes, NADPH oxidases, NADPH-cytochrome c reductase, nitric oxide synthase, and HO (1, 5, 18, 19, 27–31).

HO also appears to modulate the oxygen-sensing process of the carotid body during both normoxic and hypoxic conditions. Presumably, it is the ability of HO to catalyze the reaction of heme and oxygen to produce carbon monoxide (13) that is key to its influence on the
oxygen sensitivity of the cell. Endogenously produced carbon monoxide is now recognized as an important chemical messenger largely because of its ability to activate a host of other heme enzymes, including the stimulation of soluble guanylate cyclase (9, 32, 38). But the effect of carbon monoxide on the sensory discharge of the carotid body has been shown to be both excitatory and inhibitory. For example, Lahiri et al. (10) found that exogenously applied carbon monoxide stimulates carotid body discharge under normoxic conditions, mimicking the effect of hypoxia. In contrast, Prabhakar and colleagues (27, 28, 30), using blockers of HO, found that the endogenous production of carbon monoxide has a tonic inhibitory effect on the carotid body. These investigators went on to suggest that HO modulates the excitability of the carotid body by limiting the production of CO when oxygen is reduced, thereby promoting a disinhibition of carotid sensory discharge. Whether HO modulates the excitability of oxygen-sensitive RVLM neurons in the same manner is a possibility; however, the central effects of this chemical messenger need not mimic its peripheral pattern.

Whether and how CO modulates the excitability of central neurons appear to vary widely within the central nervous system. In the case where carbon monoxide has been used to produce progressive carboxyhemoglobinemia to limit oxygen delivery to the brain, initial increases in carboxyhemoglobin are associated with a generalized depression of respiration concomitant with an excitation of tonic sympathetic activity (21, 45). As the level of carboxyhemoglobin increases, there is a general excitation of both sympathetic and respiratory activity (45), suggesting either a threshold phenomenon and/or a neural release of the central respiratory chemosensitive elements (35, 36). Of interest is that HO has been shown to modulate the excitability of neurons in various sites within the central nervous system as well as olfactory receptor cells (8, 12, 26, 48).

Fig. 4. Immunocytochemical staining for heme oxygenase-2 in brain sections showing specific staining for nuclei and cell bodies within the RVLM. A and B: specific immunostaining of the C1 region and pre-Bötzinger complex (PreBotC) of the RVLM. C: negative control of the C1 region and PreBotC in which primary antibody was omitted from the staining protocol. D and E: specific immunostaining of the lateral reticular nucleus (LatR) localized to more caudal aspects of the RVLM. F: negative control of the LatR in which primary antibody was omitted from the staining protocol. Magnification of A and D, ×40 (calibration bar 500 μm); B and E, ×200 (calibration bar 100 μm); C and F, ×100 (calibration bar 200 μm).
ability are presumably mediated through carbon monoxide, it is important to note that areas within the brain immunoreactive for HO-2 produce measurable levels of carbon monoxide (11). Thus it seems reasonable to propose that the presence of HO in the RVLM would favor a functional role for this enzyme in modulating the excitability of cardiorespiratory neurons during changes in tissue oxygenation.

Whether the induction of HO-1 within the RVLM during sustained hypoxia plays a role in the chronic adaptations of respiration and sympathetic activity remains to be studied. However, the induction of HO-1 mRNA reflects an oxygen-sensitive change in gene expression. This increase in HO-1 gene transcription is mediated by the transcriptional regulator hypoxia-inducible-factor-1, which plays a key role in the oxygen sensitivity of a large number of genes involved in angiogenesis, energy metabolism, erythropoiesis, cell proliferation and viability, vascular remodeling, and vasomotor responses (33).

Conclusion. In conclusion, we have shown that mRNA for HO-2, the constitutive form of the enzyme, is present in the RVLM in adult rats under room air and chronic hypoxia conditions. In addition, the mRNA for the inducible form of the enzyme, HO-1, can be induced in the RVLM in rats exposed to chronic hypoxia. The expression of HO-2 within the RVLM is localized to the C1 region and pre-Bötzinger complex, areas shown in vivo to be excited by hypoxia. Localization of HO within the RVLM suggests that this enzyme may be important to the oxygen-sensing function of this region.

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