Neural Regulation of Hypoxia-Inducible Factors and Redox State Drives the Pathogenesis of Hypertension in a Rodent Model of Sleep Apnea

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Abstract

Obstructive sleep apnea (OSA) is one of the most common causes of hypertension in western societies. OSA causes chronic intermittent hypoxia (CIH) in specialized O2-sensing glomus cells of the carotid body. CIH generates increased reactive oxygen species (ROS) that trigger a feed-forward mechanism in which increased intracellular calcium levels ([Ca2+]i) trigger increased HIF-1α synthesis and increased HIF-2α degradation. As a result, the normal homeostatic balance between HIF-1α-dependent pro-oxidant and HIF-2α-dependent anti-oxidant enzymes is disrupted, leading to further increases in ROS. Carotid body sensory nerves project to the nucleus tractus solitarius, from which the information is relayed via interneurons to the rostral ventrolateral medulla in the brainstem, which sends sympathetic neurons to the adrenal medulla to stimulate the release of epinephrine and norepinephrine, catecholamines that increase blood pressure. At each synapse, neurotransmitters trigger increased [Ca2+]i, HIF-1α:HIF-2α and Nox2:Sod2 activity that generates increased ROS levels. These responses are not observed in other regions of the brainstem that do not receive input from the carotid body or signal to the sympathetic nervous system. Thus, sympathetic nervous system homeostasis is dependent on a balance between HIF-1α and HIF-2α, disruption of which results in hypertension in OSA patients.

hypoxia-inducible factors; NADPH oxidase; superoxide dismutase; oxidative stress
PATIENTS WITH OBSTRUCTIVE SLEEP APNEA (OSA) experience episodes of upper airway collapse during sleep that result in periods of hypoxemia (usually 15-30 seconds in duration) leading to arousal, clearing of the airway, and reoxygenation. These episodes of hypoxemia and reoxygenation are repeated dozens of times each night. Several large population-based studies have demonstrated that OSA is associated with an increased incidence of hypertension (11, 24). OSA is the most common secondary cause of resistant hypertension (12). Among OSA patients, the use of continuous positive airway pressure (CPAP) during sleep was associated with a reduced risk of hypertension compared to untreated OSA patients, but the incidence of hypertension was still higher than in control subjects (9).

Experimental models: Considerable progress has been made in understanding the pathogenesis of hypertension in OSA patients through the use of animal models, which have demonstrated that exposure of rodents to repetitive, brief episodes of hypoxia and reoxygenation is sufficient to induce hypertension. Thus, although OSA patients experience both chronic intermittent hypoxia (CIH) and chronic intermittent hypercapnea, CIH (in the absence of hypercapnea) is a sufficient pathophysiological stimulus to cause hypertension (2, 3) and co-exposure to both hypoxia and hypercapnia has no additional effect (8). Remarkably, the duration of the reoxygenation phase is more important than the duration of the hypoxia phase (26) and protocols in which the reoxygenation phase is longer than the hypoxia phase are more effective in eliciting hypertension than protocols with hypoxia and reoxygenation phases of equal duration (17).
Neural circuitry activated by chronic intermittent hypoxia. Bilateral transection of the carotid sinus nerve completely prevented the hypertension that was observed in sham-operated rats subjected to CIH for 35 days, demonstrating the essential role of carotid body chemoreceptors (2). The glomus cell chemoreceptors send signals via the carotid sinus nerve to the nucleus tractus solitarius, from which interneurons project to the rostral ventrolateral medulla in the brainstem (7, 22), from which efferent signals are sent via spinal nerves that synapse in sympathetic ganglia and then are transmitted via the adrenal sympathetic nerve (a branch of the splanchnic nerve) leading to stimulation of the adrenal medulla to secrete catecholamines (epinephrine and norepinephrine), which increase blood pressure (BP) (Fig. 1). Adrenal demedullation blocks the increase in BP caused by CIH (1).

Hypoxia-inducible factors mediate transcriptional responses to continuous hypoxia. The hypoxia-inducible factors HIF-1 and HIF-2 control cellular and systemic responses to continuous hypoxia (18). HIFs are heterodimeric transcription factors composed of an O2-regulated HIF-1α or HIF-2α subunit and a constitutively expressed HIF-1β subunit. Whereas HIF-1α and HIF-1β are found in all metazoans, HIF-2α is only found in vertebrates, in which increase in body mass necessitated the evolution of complex cardiovascular and respiratory systems in order to insure adequate tissue O2 delivery (21). Homozygosity for a null allele at the locus encoding HIF-1α or HIF-2α results in embryonic or postnatal lethality, respectively (4, 20).

Heterozygous HIF-1α-null (Hif1a) mice develop normally and are indistinguishable from wild type (WT) littermates under normoxic conditions but have impaired responses to hypoxia and ischemia (25). Carotid bodies isolated from Hif1a
mice do not respond to acute hypoxia, although the response to cyanide or hypercarbia remains intact and histology of the carotid body is normal, indicating a specific defect in the ability to sense or respond to hypoxia (6). In contrast, carotid bodies isolated from heterozygous HIF-2α-null (Epas1) mice manifest exaggerated responses to acute hypoxia (13).

**Molecular circuitry activated by chronic intermittent hypoxia in the carotid body.**

To model CIH experienced by patients with OSA, Hif1a and WT littermate mice were placed in an environmental chamber in which the O2 concentration was rapidly dropped from 21% to 5% for 15 seconds and then rapidly increased to 21% for 5 minutes, and this hypoxia-reoxygenation cycle was repeated for 8 hours per day over 10 days, which caused an increase in mean BP from ~110 to >140 mmHg in WT mice but no significant BP increase in Hif1a littermates (15). The increase in BP was associated with a significant increase in plasma catecholamine levels in WT mice subjected to CIH, but not in Hif1a littermates (15). Whereas Hif1a mice with partial HIF-1α deficiency fail to develop hypertension in response to CIH, Epas1 mice with partial HIF-2α deficiency manifest hypertension even under normoxic conditions (13).

Analysis of WT mice exposed to CIH revealed that HIF-1α levels increased in the carotid body (15), whereas HIF-2α levels decreased (10). The cycles of hypoxia and reoxygenation during CIH cause an increase in reactive oxygen species (ROS) (16). ROS generated during CIH increase intracellular calcium levels [Ca2+]i, leading to protein kinase C (PKC)-dependent induction of mTOR (mammalian target of rapamycin) activity that increased HIF-1α expression (15, 28), which led to increased expression of the Nox2 gene encoding the pro-oxidant enzyme NADPH oxidase (27). ROS generated by Nox2
has been shown to interfere with the normal function of mitochondrial electron transport complex I, resulting in a further increase in ROS generation (5). The ROS-dependent increase in [Ca\(^{2+}\)], also activated calpains, which are calcium-dependent proteases that mediate the degradation of HIF-2\(\alpha\), thereby preventing it from activating transcription of the Sod2 gene, which encodes a mitochondrial isoform of the anti-oxidant enzyme superoxide dismutase (10).

Not only did HIF-1\(\alpha\) and HIF-2\(\alpha\) have opposing effects on the cellular redox state, they actively antagonized each other: in Epas1 mice, decreased Sod2 activity resulted in a pro-oxidant state that induced HIF-1\(\alpha\) and Nox2 expression under normoxic conditions, whereas in Hif1a mice, decreased Nox2 expression and a reduced redox state blocked the degradation of HIF-2\(\alpha\), leading to increased Sod2 expression (28). Most remarkably, Epas1;Hif1a compound heterozygous mice, which are partially deficient for both HIF-1\(\alpha\) and HIF-2\(\alpha\), had completely normal BP and carotid body responses to hypoxia (29), demonstrating that it is the ratio of HIF-1\(\alpha\) to HIF-2\(\alpha\) that determines the set point of carotid body reactivity, thereby controlling sympathetic activation and BP (Fig. 2).

*Intermittent hypoxia is transduced to intermittent neural activity.* In addition to the remarkable finding that CIH increased HIF-1\(\alpha\) and Nox2 expression, decreased HIF-2\(\alpha\) and Sod2 expression, and increased oxidative stress in glomus cells of the carotid body, the same changes were observed in neurons of the nTS and RVLM, but not in nearby brainstem regions that do not receive input from the carotid body, as well as in chromaffin cells of the adrenal medulla (14). These findings suggested either that cells in the nTS, RVLM, and adrenal medulla directly sense and respond to CIH, in a manner
similar to the carotid body, or that neural activity induces the same response in these cells that are induced by CIH in the carotid body.

To test whether carotid body neural activity is required for downstream effects, rats were subjected to carotid body ablation. CIH induced increased HIF-1α protein and Nox2 activity and decreased HIF-2α protein and Sod2 activity in nTS, RVLM, and adrenal medulla of control rats but not in rats subjected to carotid body ablation (14). CIH induced increased adrenal sympathetic nerve activity in response to a subsequent episode acute hypoxia and this response was lost in rats subjected to carotid body ablation; responses in the adrenal medulla were also lost in rats subjected to adrenal sympathetic nerve ablation (14).

In the adrenal medulla, sympathetic nerves release acetylcholine, which binds to muscarinic or nicotinic acetylcholine receptors on the surface of the catecholamine-secreting chromaffin cells. Treatment of rats with atropine during a 10-day exposure to CIH had no effect on carotid body or adrenal sympathetic nerve activity but blocked the response of the adrenal medulla to CIH, indicating that muscarinic acetylcholine receptors mediate the effects of the sympathetic nervous system on chromaffin cells of the adrenal medulla (14).

To further analyze the response of chromaffin cells to sympathetic activation, the rat PC12 pheochromocytoma cell line, which was originally established from a tumor of rat adrenal medullary chromaffin cells, was studied. Repetitive, intermittent application of muscarine (5-minute treatment followed by 10-minute washout for 3 cycles) was sufficient to increase HIF-1α and decrease HIF-2α protein levels, and the effect was blocked by atropine. In contrast, repetitive, intermittent application of nicotine, or
constant application of muscarine or nicotine, had no effect (14). Repetitive, intermittent
muscarine application increased \([\text{Ca}^{2+}]_i\) and mTOR activity and these responses, which
were blocked by atropine, were required for induction of HIF-1\(\alpha\) protein expression
because muscarine did not induce HIF-1\(\alpha\) expression in the presence of the calcium
chelator BAPTA-AM or the mTOR inhibitor rapamycin. Repetitive, intermittent
muscarine treatment of PC12 cells also increased calpain activity and the calpain
inhibitor Ac-LLM-CHO blocked HIF-2\(\alpha\) degradation that was induced by muscarine
treatment (14). Consistent with the PC12 studies, analysis of adrenal medulla from rats
subjected to CIH revealed increased activity of mTOR and calpains, which provide a
basis for increased HIF-1\(\alpha\) and decreased HIF-2\(\alpha\) levels, respectively, and these effects
were blocked by carotid body ablation, adrenal sympathetic nerve ablation, or atropine
treatment (14).

As expected, the increase in plasma catecholamines and BP that are induced by
CIH were not observed in rats that were subjected to carotid body ablation or adrenal
sympathetic nerve ablation. These results demonstrate that the response of the adrenal
medulla is dependent of transmission of neural signals generated in the carotid body in
response to CIH. Remarkably, unlike adrenal medulla from control rats, which is
unresponsive, sections of adrenal medulla from CIH-exposed rats increased
catecholamine secretion when subjected to hypoxia \emph{ex vivo} (14). It is also interesting that
in PC12 cells, increased HIF-1\(\alpha\) \(\rightarrow\) Nox2 and decreased HIF-2\(\alpha\) \(\rightarrow\) Sod2 signaling can
be induced either by intermittent hypoxia (10, 14) or repetitive, intermittent muscarine
application (14), highlighting the interconversion of ROS signals and neural activity that
is mediated by changes in the ratio of HIF-1\(\alpha\) to HIF-2\(\alpha\).
Summary. Studies in rodents indicate that the relative steady state levels of HIF-1α and HIF-2α protein in O2-sensing glomus cells of the carotid body determine the set point of sympathetic nervous system activity and blood pressure in healthy individuals. In patients with OSA, CIH increases ROS, which trigger increased \([Ca^{2+}]_i\), leading to increased PKC-dependent mTOR activity and increased calpain activity, which cause increased HIF-1α and decreased HIF-2α levels, leading to increased Nox2 and decreased Sod2 activity that together further increase ROS in the carotid body in a feed-forward mechanism (Fig. 2). Increased \([Ca^{2+}]_i\) also results in the depolarization of glomus cells leading to increased activity of neurons that project to the nTS and from there to the RVLM in the brainstem, where they synapse with sympathetic neurons that project via the spinal cord to sympathetic ganglia, where they synapse with neurons that project via the adrenal sympathetic nerve to activate muscarinic receptors on chromaffin cells in the adrenal medulla and stimulate their release into the circulation of epinephrine and norepinephrine, which are catecholamines that cause vasoconstriction and thereby increase BP (Fig. 1). Clinical studies have also demonstrated impaired endothelium-dependent vasodilation in OSA patients (19), but it is not known whether this defect is a direct effect of CIH on endothelial cells or a secondary effect of increased circulating catecholamines.}

Whereas glomus cells in the carotid body respond directly to ROS resulting from CIH, neurons in the nTS, RVLM, and adrenal medulla depolarize in response to neurotransmitters and the resulting increased \([Ca^{2+}]_i\) triggers an increase in HIF-1α:HIF-2α expression and Nox2:Sod2 activity that results in ROS generation as well as signal transmission that ultimately results in catecholamine release and hypertension (Fig. 2).
Thus, sympathetic nervous system homeostasis is dependent on a balance between HIF-1α and HIF-2α levels, the disruption of which results in oxidative stress and hypertension. These studies have delineated the molecular and cellular mechanisms that underlie the most common cause of treatment-resistant hypertension in western society. Drugs that inhibit HIF-1α but not HIF-2α, such as ganetespib, which is currently being evaluated in clinical trials as an anti-cancer drug (23), may be of therapeutic benefit to hypertensive OSA patients who do not respond to CPAP or standard anti-hypertensive agents.

Finally, independent of OSA, the balance between HIF-1α and HIF-2α establishes a redox balance in the carotid body, which in turn establishes a set point for sympathetic nervous system activity, which is a major determinant of blood pressure. This fundamental physiological role of antagonism between HIF-1α and HIF-2α in the carotid body is highlighted by the phenotype of Epas1+/- mice, in which reduced HIF-2α expression leads to oxidative stress and increased HIF-1α expression in the carotid body and adrenal medulla that causes hypertension under normal ambient environmental conditions. Other genetic or environmental factors that alter the balance between HIF-1α and HIF-2α in the carotid body may also result in hypertension.

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DISCLOSURES

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Fig. 1. Neural circuitry by which chronic intermittent hypoxia increases catecholamine secretion to induce hypertension. Experimental manipulations that are discussed in the text (CBA, ASA, and ATR) are shown by vertical bars. Abbreviations: CSN, carotid sinus nerve; CBA, carotid body ablation; SC, spinal cord; ASN, adrenal sympathetic nerve; ASA, ASN ablation; ATR, atropine.

Fig. 2. Molecular mechanisms leading to activation of glomus cells in the carotid body in response to chronic intermittent hypoxia. HIF, hypoxia-inducible factor; NOX, NADPH oxidase; SOD, superoxide dismutase; \([\text{Ca}^{2+}]_i\), intracellular calcium ion concentration; PKC, protein kinase C; mTOR, mammalian target of rapamycin.
Chronic Intermittent Hypoxia

HIF-1α → NOX2 → ROS | SOD2 ← HIF-2α

[Ca^{2+}]_i

PKC mTOR

Calpain

Carotid Body Activation

Sympathetic Nervous System Activation

Systemic Hypertension

Figure 2