Endothelin-1 mediates intermittent hypoxia-induced inflammatory vascular remodeling through HIF-1 activation

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Gras E, Belaidi E, Briançon-Marjollet A, Pépin J-L, Arnaud C, Godin-Ribuot D. Endothelin-1 mediates intermittent hypoxia-induced inflammatory vascular remodeling through HIF-1 activation. J Appl Physiol 120: 437–443, 2016. First published December 17, 2015; doi:10.1152/japplphysiol.00641.2015.—Obstructive sleep apnea (OSA) syndrome is a common sleep-related breathing disorder characterized by repetitive upper airway collapse during sleep resulting in intermittent hypoxia (IH), sleep fragmentation, and obstructed respiratory efforts (36). OSA represents a substantial public health problem because it affects up to 10% of the general population and is recognized as an important and independent risk factor for cardiovascular disease (36). Accumulating evidence from patients with OSA and animal models suggests that chronic IH is the most important OSA-related stimulus in terms of cardiovascular morbidity and mortality (10).

OSA is an independent risk factor for atherosclerosis (2), and IH is a major contributing factor to atherosclerosis because its progression correlates with the severity of nocturnal oxygen desaturation (4, 27) and is reversed by continuous positive airway pressure (11). Patients with OSA exhibit increased carotid intima-media thickness (IMT), an early sign of atherosclerosis (12), which correlates with serum inflammatory markers and oxygen desaturation (4, 27). Moreover, patients with OSA exhibit elevated plasma levels of prothrombotic and proinflammatory factors that also correlate with oxygen desaturation (29) and are major determinants of atherosclerosis (39).

The last few decades, a large body of evidence has indicated that the vasoactive peptide endothelin-1 (ET-1) plays a major role in vascular inflammation and subsequent atherosclerosis progression. In human vascular smooth muscle cells (VSMC) it has been shown that ET-1 activates the proinflammatory transcription factor nuclear factor-κB (NF-κB) and subsequently induces the release of cytokines such as IL-6 (8). In the same manner, Verma et al. (40) demonstrated that the vascular proinflammatory effect of C-reactive protein is in part related to the increased production of ET-1 (40). In addition to its role in vascular inflammation, ET-1 is known to stimulate VSMC proliferation and migration, and synthesis of extracellular matrix and matrix remodeling (18), thus supporting its contributing role in vascular remodeling and atherosclerosis.

Some studies in patients with OSA have demonstrated an increase in plasma ET-1 release (16, 31). Using chronic IH (i.e., to mimic the major consequence of OSA), we and others have demonstrated that IH strongly increases the tissue and plasma levels of ET-1, which is a key factor in the development of IH-induced hypertension (22) and IH-dependent increase in myocardial infarct size (6). It has been proposed that IH increases ET-1 synthesis through increased production of reactive oxygen species. This subsequently activates the nuclear factor of activated T cells (NFATc3), which then increases vasoconstrictor reactivity and calcium response to ET-1 (14). To date, most studies have focused on IH-induced increased vasoconstrictive response to ET-1 [for a review see (21)], and only one showed the contribution of ET-1 to IH-induced arterial remodeling (9). The underlying mechanism of ET-1 involvement in IH-related inflammation and vascular remodeling remains unclear.

Among the mechanisms known to regulate ET-1 bioavailability is the rate of transcription of the ET-1 gene (edn1) (37),
which is upregulated under the control of hypoxia-inducible factor-1 (HIF-1) (26). Using chromatin immunoprecipitation, we have previously demonstrated that IH promotes the binding of HIF-1 to the edn1 gene promoter in the heart (6). In addition, HIF-1, which is a major transcription factor involved in the response to hypoxia (35), is also known to play a role in vascular wall disease (25) and regulation of intrinsic immune and inflammatory responses (28). Furthermore, it has been well established that an intimate crosstalk exists between HIF-1 and NF-kB under hypoxic conditions (38) and, as mentioned above, ET-1 is a major contributor to vascular inflammation and atherosclerosis, in particular through activation of NF-kB and subsequent release of cytokines (8).

Thus the aim of the present work was to investigate whether ET-1 promotes IH-induced vascular inflammation and arterial remodeling through HIF-1-dependent NF-kB activation.

METHODS

Animals

All experiments were conducted in accordance with the European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes (Council of Europe, European Treaties ETS 123, Strasbourg, March 18, 1986) and the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996), and were approved by the animal care and use committee of the Université Grenoble Alpes.

The role of the HIF-1/ET-1 axis in IH-related inflammation and associated vascular remodeling was investigated in mice 7 to 9 wk old that were partially deficient for HIF-1α in their wild-type littermates (50% Swiss/50% 129SvJ), and in C57BL/6J mice treated or not with the nonselective ET-1 receptor antagonist bosentan (100 mg·kg⁻¹·day⁻¹, added to the food) graciously provided by Actelion (Basel, Switzerland).

Intermittent Hypoxia and Samples Collection

Mice were randomized and submitted to IH as previously described (10). Briefly, animals were exposed during their daytime sleeping period to 8 consecutive hours of 1-min cycles (alternating 30-s periods of 21% and 5% FiO2) for 14 consecutive days. FiO2 was monitored throughout the experiments with a gas analyzer (ML206; ADInstruments, Oxford, U.K.). Control animals in normoxia (N) experiments were exposed to similar 1-min air cycles to reproduce the noise and air turbulence of the IH stimulus. After 14 days of exposure, mice were anesthetized with ketamine and xylazine (100 and 10 mg/kg, respectively). Blood was obtained by cardiac puncture and collected in tubes containing EDTA (K2E; BD Microtainer, Pont de Claix, France) for hematocrit assessment and cytokine assay. Thoracic aorta were removed and either embedded in optimum cutting temperature (OCT) compound (Tissue tek-OCT; Sakura, Ensart, Belgium) using ELISA DNA-binding assay kits (TransAM, Active Motif Europe).

Aortic Hydroxyproline and Desmosine Assay

A 3-mm piece of thoracic aorta was used to determine aortic wall composition. Tissues were lysed in HCl vapor and the lysate was resuspended in milli-Q water. Desmosine levels, representative of elastin content, were quantified by radioimmunoassay as described by Starcher and Conrad (36a). Hydroxyproline levels, representative of collagen content, were assessed by ion-exchange chromatography using the Biochrom 30 amino acid analyzer (Cambridge, UK).

Splenocyte Activation

Splenocyte proliferation. Splenocytes were isolated and cultured in 96-well plates at a concentration of 5 × 10⁶ cells/ml in RPMI 1640 supplemented with 10% FBS, 25 mM HEPES buffer, 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cells were stimulated in triplicates with graded concentrations of the mitogenic factor concanavalin-A (Con-A) (Sigma, Saint-Quentin-Fallavier, France) and cell proliferation was determined 48 h later using a nonradioactive MTS cell proliferation assay (Promega, Charbonnières-les-Bains, France).

Splenocyte cytokine secretion. For cytokine secretion, cultured splenocytes were stimulated with 2 µg/ml of Con-A. Supernatants were recovered after 48 to 72 h, after which murine IFN-γ, IL-4, and IL-10 were assayed by ELISA using paired antibodies according to the manufacturer’s instructions (R&D Systems, Lille, France).

Intima-Media Thickness

OCT-embedded 8-µm aortic sections were stained with hematoxylin-eosin. Measurements were carried out under light microscopy (Eclipse 80i; Nikon Instruments, Champigny-sur-Marne, France) using the NIS-Element AR 3.10 analysis software (Nikon Instruments). Morphometric analysis was performed on 8 to 10 aortic sections per animal (up to 10 measurements per section).

Aortic HIF-1 and NF-κB p50 Activity

HIF-1 and NF-κB p50 activities were quantified on nuclear protein extracts (Nuclear Extract Kit; Active Motif Europe, Rixensart, Belgium) using ELISA DNA-binding assay kits (TransAM, Active Motif Europe).

Aortic Cytokine Assay

Cytokine quantification was performed using a commercial kit (AAM-CYT-2-4; RayBiotech, Norcross, GA) according to the manufacturer’s instructions. Protein (250 µg, pooled from two different extracts) was loaded on a membrane containing 32 cytokine antibodies (one membrane per condition). Briefly, membranes were blocked for 30 min at room temperature (RT). Samples were incubated overnight at 4°C. Membranes were then washed and incubated for 2 h at RT with a biotinylated antibody cocktail followed by incubation for 2 h at RT with horseradish peroxidase-streptavidin. Finally, chemiluminescence was detected using the Chemidoc XRS+ system (Biorad, Marne-la-coquette, France). Analysis was performed with ImageLab software (Biorad) and the results were expressed relative to the normalized value of the normoxia group.

Statistical Analysis

Experimental data are presented as means ± SE. Data were analyzed using two-way ANOVA followed by Bonferroni post hoc comparisons or Mann-Whitney tests (Prism, GraphPad software, La Jolla, CA). A two-sided value of P < 0.05 was considered statistically significant.

RESULTS

ET-1 and HIF-1 Contribute to IH-Induced Systemic Inflammation

Although there was no difference in plasma cytokine levels between normoxic and hypoxic animals (data not shown), mice exposed to 14 days of IH displayed an increase in splenocyte proliferative capacities (Fig. 1A). This was accompanied by a significant increase in IFN-γ secretion (Fig. 1C). The IH-induced increase in splenocyte proliferative and secretory capacity (Fig. 1, B and C, respectively) was prevented by bosen-
tan treatment. IL-4 and IL-10 secretions (Fig. 1, D and E, respectively) were not affected by IH or by bosentan treatment.

As observed in C57BL/6J mice, the proliferative response to Con-A was also increased in splenocytes from HIF-1α+/+ mice exposed to IH compared with N (Fig. 2A), and this was abolished in mice with HIF-1α partial deficiency (Fig. 2B).

*ET-1 and HIF-1 Are Involved in IH-Induced Structural Aorta Remodeling*

Histological examination of the aortic wall revealed that IH resulted in structural vascular remodeling in both C57BL/6J and HIF-1α+/+ mice as characterized by greater IMT (27.8 ± 0.8 vs. 21.7 ± 0.6 µm in C57BL/6J mice exposed to IH and N, respectively, *P* < 0.05, Fig. 3A; 34.6 ± 0.8 vs. 28.5 ± 0.8 µm in HIF-1α+/+ mice exposed to IH and N, respectively, *P* < 0.05, Fig. 3B). All IH-induced alterations of the vascular wall were prevented by bosentan treatment and partial HIF-1α deficiency (Fig. 3, A and B). This IH-increased IMT was accompanied by signs of disorganization and discontinuity of elastic fibers (Fig. 3C). This remodeling was not related to higher levels of elastin or collagen content, as reflected by aortic desmosine and hydroxyproline levels, respectively (data not shown).

*ET-1 Promotes IH-Induced NF-κB Activation and Aortic Inflammation*

Aorta from C57BL/6J mice exposed to IH also exhibited an increase in NF-κB activity (125.2 ± 5.6 vs. 100.0 ± 4.3% of normoxic group value in mice exposed to IH and N, respectively, *P* < 0.05), which was prevented by bosentan treatment.
ET-1 and HIF-1 are involved in IH-induced structural aortic remodeling. A: histomorphometric analysis of thoracic aorta intima-media thickness (IMT) in mice untreated or treated with bosentan (100 mg·kg⁻¹·day⁻¹) and exposed to 14 days of IH or N. *P < 0.05 vs. N (n = 5–6 per group). B: histomorphometric analysis of IMT in HIF-1⁺/⁺ and HIF-1⁻/⁻ mice exposed to 14 days of IH or N. *P < 0.05 vs. N (n = 9–13 per group). C: representative photographs of hematoxylin- eosin staining (×10 magnification) of thoracic aorta from mice exposed to N (top) or IH (bottom) for 14 days.

**DISCUSSION**

This study provides further insight into the mechanisms behind the inflammatory vascular remodeling induced by IH in mice. We demonstrated for the first time that the HIF-1 transcription factor is required for IH-induced systemic and vascular proinflammatory effects. We further showed that ET-1 also participates in IH-induced HIF-1 activation and aortic inflammatory remodeling through NF-κB activation. This provides a strong rationale for considering ET-1 receptor blockade as a new therapeutic target when addressing the vascular consequences of OSA.

**ET-1 and HIF-1 Are Required for IH-Induced Low-Grade Systemic Inflammation and Aortic Structural Remodeling**

**Systemic inflammation.** Spleen-derived T-cell activation (i.e., proliferation and cytokine secretion) in response to Con-A represents an index of systemic T-lymphocyte activation (1). We investigated whether ET-1 could also be involved in IH-induced chronic low-grade systemic inflammation and showed that treatment with bosentan (a mixed ET-1 receptor antagonist) throughout IH exposure abolished the IH-induced proliferative and secretory capacities of splenocytes. In addition, HIF-1 also appears to play a major role in IH-associated chronic low-grade systemic inflammation because we observed that partial HIF-1α deletion was sufficient to completely prevent IH-induced splenocyte proliferation. Taken together, our results demonstrate that ET-1 and HIF-1 contribute to IH-associated low-grade chronic systemic inflammation. In a previous study using chromatin immunoprecipitation, we demonstrated that IH promotes the binding of HIF-1 to the edn1 gene promoter in rats (6), and a very recent study demonstrated that endothelin-converting enzyme (ECE-1), a plausible HIF-1 target gene (23), is also activated by IH (30). This suggests that HIF-1-associated ET-1 system regulation could be involved in IH-related inflammatory effects.

**Structural aortic remodeling.** An increase in carotid IMT has been well described in patients with OSA (4, 12) and has been correlated with both circulating levels of inflammatory markers and oxygen desaturation (4, 27). In our murine model, exposure of mice to 14 days of IH resulted in enlarged IMT, which is in agreement with previous studies in mice showing mechanical adaptations of the arterial wall to IH (1, 9, 32). The IH-associated increase in IMT, which is indicative of an early vascular remodeling, was prevented by both bosentan treatment and partial deletion of HIF-1α. These findings taken together confirm an important role for ET-1 and HIF-1 in vascular remodeling induced by IH.

**IH-Induced Inflammation of Aorta Involves ET-1 and HIF-1**

In the present study, we showed that IH induces activation of NF-κB in the aorta, which is in accordance with previous studies by our group (1, 32) and others (17), and we further demonstrated that bosentan treatment blunted both this response and the IH increase in aortic cytokine expression (MCP-5, CTACK, THPO). These results demonstrate that ET-1 promotes IH-induced inflammation and are in agreement with those from a previous study showing that ET-1 directly promotes inflammation through NF-κB activation in human VSMC (8). Some studies have also demonstrated an increase in...
plasma ET-1 content in patients with OSA (16, 31) and an increase in ET-1 in rodents exposed to IH (6, 22). De Frutos et al. (9) described the involvement of ET-1-induced activation of nuclear factor of activated T cells isoform 3 (NFATc3) as a potential mechanism behind the IH-associated vascular remodeling (9). In the present study we demonstrated for the first time that ET-1 is also involved in IH-related activation of NF-κB activity in aorta and associated vascular remodeling.

Although we previously demonstrated that IH susceptibility to ET-1 in coronary arteries and its involvement in the development of hypertension were mediated by HIF-1 (6), we highlight here that ET-1 could in turn be involved in HIF-1 activation.

Fig. 4. ET-1 promotes IH-induced nuclear factor-κB (NF-κB) activity and aorta inflammation. NF-κB p50 activity in thoracic aorta of mice untreated or treated with bosentan (100 mg·kg⁻¹·day⁻¹) in response to 14 days of exposure to IH or N. *P < 0.05 vs. N (n = 15 per group). A: cytokine expression in thoracic aorta of mice untreated and treated with bosentan (100 mg·kg⁻¹·day⁻¹) and exposed to 14 days IH or N. B: cytokines overexpressed by IH and not affected by bosentan included granulocyte macrophage colony-stimulating factor (G-CSF); granulocyte macrophage colony-stimulating factor (GM-CSF); interleukin, IL; thymus and activation regulated chemokine, TARC; and tissue inhibitor of metalloproteinases, TIMP-1. C: cytokines overexpressed by IH and decreased by bosentan included monocyte chemotactic protein-5, MCP-5; tumor necrosis factor-α, TNF-α; thrombopoietin, THPO; and cutaneous T-cell attracting chemokine, CTACK.

Fig. 5. ET-1 promotes IH-induced NF-κB activity through HIF-1 activation. A: NF-κB activity in thoracic aorta of HIF-1⁺/⁺ and HIF-1⁻/⁻ mice exposed to 14 days of IH or N; *P < 0.05 vs. N (n = 6–7 per group). B: HIF-1 activity in thoracic aorta of mice either untreated or treated with bosentan (100 mg·kg⁻¹·day⁻¹) and exposed to 14 days of IH or N; *P < 0.05 vs. N (n = 9–12 per group).
activation. Furthermore, HIF-1 is known to closely interact with NF-κB (28), and this crosstalk has been well described in several conditions. HIF-1 is known to activate NF-κB in hypoxic neutrophils (41) and, conversely, NF-κB is needed for the hypoxia-induced upregulation of HIF-1α transcription in pulmonary artery smooth muscle cells (5) or for HIF-1α protein accumulation in liver and brain of hypoxic animals (34). In our study we showed a similar link between HIF-1 and NF-κB, because IH-induced aortic NF-κB activation was blunted in HIF-1α−/− mice. Thus HIF-1 seems to exert a promoting role on IH-induced NF-κB activation.

In conclusion, IH-induced thickening of the aortic wall is accompanied by proinflammatory effects that appear to be caused principally by ET-1. We described an amplifying loop in which ET-1 leads to early inflammatory remodeling by activating NF-κB, probably through an HIF-1-dependent pathway. Indeed, both partial HIF-1α deficiency and endothelin receptor antagonist antagonism were sufficient to completely reverse the systemic and vascular inflammatory effects of IH and associated vascular remodeling. These arguments are in favor of a therapeutic approach targeting ET-1 receptors in patients with OSA at risk for vascular disease.

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REFERENCES


