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Selective inhibition of endothelial NF-κB signaling attenuates chronic intermittent hypoxia-induced atherosclerosis in mice

Dongmei Song [a, b *], Guoqiang Fang [a], Sun-zhong Mao [a], Xiaobing Ye [a], Gang Liu [a], Edmund J. Miller [a], Harly Greenberg [a], Shu Fang Liu [a, b, **]

[a] Center for Pulmonary, Critical Care and Sleep Medicine, The Feinstein Institute for Medical Research, Donald and Barbara Zucker School of Medicine at Hofstra/Northwell, Manhasset, NY, 11030, USA
[b] The First Affiliated Hospital of Hebei Medical University, Shijiazhuang, 050031, Hebei, China

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ABSTRACT

Background and aims: Chronic intermittent hypoxia (CIH) exposure causes atherosclerosis, although the underlying mechanisms are poorly understood. This study defines the role of endothelial intrinsic NF-κB signaling in the atherogenic response to CIH.
Methods: We created ApoE-EC$^{I\text{-}\kappa Bmt}$ mice that are deficient in the apolipoprotein E gene ($ApoE^{-/-}$) and overexpress an I-κBα mutant (I-κBmt) selectively in endothelial cells. $ApoE^{-/-}$ and ApoE-EC$^{I\text{-}\kappa Bmt}$ mice were fed a normal chow diet (NCD) or high cholesterol diet (HCD) and exposed to sham or CIH, and atherosclerotic lesions were quantified.
Results: CIH exposure activated NF-κB in aortas, and induced the expression of endothelial-specific and NF-κB-dependent genes, E-selectin and vascular cell adhesion molecule (VCAM)-1, in the aortas and hearts. Endothelial I-κBmt overexpression in ApoE-EC$^{I\text{-}\kappa Bmt}$ mice significantly inhibited CIH-induced NF-κB activity, and suppressed E-selectin and VCAM-1 expressions, confirming endothelial NF-κB inhibition in ApoE-EC$^{I\text{-}\kappa Bmt}$ mice. $ApoE^{-/-}$ mice, on NCD, developed mild atherosclerotic lesions spontaneously, and developed advanced and larger areas of atherosclerotic plaques when exposed to CIH. $ApoE^{-/-}$ mice also developed advanced atherosclerotic lesions when fed an HCD alone. The HCD-induced atherosclerotic plaques became more advanced, and plaque area was doubled in mice exposed to HCD + CIH. Endothelial I-κBmt overexpression in ApoE-EC$^{I\text{-}\kappa Bmt}$ mice attenuated spontaneously developed atherosclerotic lesions, abrogated CIH-induced atherosclerosis and mitigated CIH-mediated facilitation of HCD-induced atherosclerosis.
Conclusions: These results suggest that endothelial intrinsic NF-kB signaling may play a pivotal role in CIH-induced atherosclerosis.

1. Introduction

Chronic intermittent hypoxia (CIH), a prominent feature of obstructive sleep apnea (OSA) [1,2], is characterized by repetitive episodes of hypoxia followed by normoxia (reoxygenation). In patients with OSA, this episodic CIH occurs tens, even hundreds of times per night for years [1,2], resulting in an increased oxidant stress and increased risk of cardiovascular diseases [3–8], including coronary and cerebral vascular diseases, and stroke [4–8], common consequences of atherosclerosis.

Emerging evidence suggests that CIH exposure is an important risk factor for atherosclerosis [9–11]. Compared to age- and body mass index-matched controls, patients with OSA had increased carotid artery intima-media thickness and carotid to femoral artery pulse wave velocity, early signs of atherosclerosis, [11–14]. Those changes were reduced by treatment with continuous positive airway pressure to correct the OSA [12]. Animal studies show that CIH is an independent causal factor of atherosclerosis. Apolipoprotein E gene knockout ($ApoE^{-/-}$) mice with no pre-existing atherosclerotic lesions and fed a normal chow diet (NCD) developed early or advanced atherosclerotic lesions following 9 or 30

* Corresponding author. The First Affiliated Hospital of Hebei Medical University, 89 Dong Gang Road, Shijiazhuang, 050031, Hebei Province, China.
** Corresponding author. The Feinstein Institute for Medical Research, 350 Community Drive, Manhasset, NY 11030, USA.
E-mail addresses: dongmeisong2016@126.com (D. Song), SLiu@northwell.edum (S.F. Liu).

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weeks of exposure to CIH [9]. CIH exposure facilitates high cholesterol diet (HCD)-induced atherosclerosis. Wild type mice did not develop atherosclerosis when fed an HCD or exposed to CIH alone, but developed atherosclerotic lesions when exposed to HCD + CIH [15,16]. *ApoE*$^{-/-}$ mice exposed to HCD + CIH had larger atherosclerotic plaque areas and bigger plaque size, compared to mice fed with an HCD alone [17–19]. CIH exposure accelerates the progression of existing atherosclerotic lesions. In *ApoE*$^{-/-}$ mice with established atherosclerotic lesions (15–20 weeks old) and on an NCD, mice exposed to CIH showed larger and more advanced atherosclerotic plaques, compared to mice subjected to sham exposure [20–22]. Thus, CIH exposure could be a key pathogenic factor underlying the association between OSA and atherosclerosis. However, the mechanisms and pathways mediating CIH-induced atherosclerosis remain largely unknown.

Activation of the NF-κB pathway has been implicated in the pathogenesis of atherosclerosis in the HCD-induced atherosclerosis model. NF-κB activity increased in all major cell types in the atherosclerotic plaques [23]. Deletion of NF-κB target genes alleviated HCD-induced atherosclerosis [23]. Systemic inhibition of NF-κB protected against HCD-induced atherosclerosis [24,25]. The role of cell-intrinsic NF-κB activity in HCD-induced atherosclerosis varies depending on the cell types studied. Selective blockade of NF-κB activity in endothelial cells [26] or hematopoietic cells [27] alleviated, whereas selective inhibition of NF-κB activity in macrophages exacerbated, HCD-induced atherosclerosis [28]. Overall, activation of the NF-κB pathway plays an important, but complex role in HCD-induced atherosclerosis.

There are major differences between CIH- and HCD-induced atherosclerosis. CIH-induced atherosclerotic plaque contains a large number of CD31^{+} endothelial cells and has mainly elastin deposition, whereas HCD-induced atherosclerotic plaque contains few CD31^{+} cells and has mainly collagen deposition [10]. CIH does not cause macrophage foam cell formation *in vivo* [10], whereas macrophage foam cell formation is a major feature of HCD-induced atherosclerosis. Thus, CIH and HCD may induce atherosclerosis by distinctive mechanisms. The NF-κB pathway, particularly cell-intrinsic NF-κB signaling, may play a different role in CIH-induced atherosclerosis. However, the role of endothelial NF-κB signaling in CIH-induced atherosclerosis has not been previously studied.

The aim of this study was to define the role of endothelial NF-κB signaling in CIH-induced atherosclerosis. We created ApoE-EC$^{I\text{-}\kappa Bmt}$ triple mutant mice that are deficient in apolipoprotein E gene and overexpress a doxycycline (Dox)-inducible I-κBα mutant (I-κBmt) selectively in endothelial cells. Using this mouse model, we demonstrated that endothelial intrinsic NF-κB signaling may play an important role in CIH-induced atherosclerosis.

2. Materials and methods

2.1. Animals

All animal study protocols were approved by the Institutional Animal Care and Use Committee of the Feinstein Institute for Medical Research, and were carried out in accordance with the Guide for Care and Use of Laboratory Animals of the NIH. Two transgenic mouse strains were initially created and characterized [29]. The EC^{rtTA} mice overexpress the tetracycline-dependent transactivator (rtTA) under the control of an endothelial-specific VE-cadhein-5 promoter. Tre-I-κBmt mice overexpress I-κBαmt under the control of the Tre-CMV fusion promoter, whose activation depends on the binding of rtTA and Dox. *ApoE*$^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, ME). To generate ApoE-EC^{rtTA} and ApoE-Tre-I-κBmt mice, the EC^{rtTA} and Tre-I-κBmt mice were back-crossed into the *ApoE*$^{-/-}$ genetic background for 6 generations and bred to homozygosity for both genes (ApoE and rtTA or ApoE and I-κBmt). Subsequently, ApoE-EC$^{I\text{-}\kappa Bmt}$ mice were created by cross-breeding between ApoE-EC^{rtTA} and ApoE-Tre-I-κBmt mice.

Genotyping for rtTA and Tre-I-κBmt alleles was carried out using Southern blot analysis as we previously described [29]. Homozygous and heterozygous rtTA or Tre-I-κBmt alleles were distinguished based on rtTA or Tre-I-κBmt band intensity. ApoE genotyping was performed by PCR following the protocol designed by Jackson Laboratory. The primer set amplifies a single band of 155 bps in genomic DNAs (gDNAs) from *ApoE*$^{+/+}$ mice, a single band of 245 bps in gDNAs from homozygous *ApoE*$^{-/-}$ mice, or double bands of 155 and 245 bps in gDNAs from heterozygous *ApoE*$^{+/-}$ mice.

To confirm that the ApoE-EC$^{I\text{-}\kappa Bmt}$ mice express Dox-inducible I-κBmt, ApoE-EC$^{I\text{-}\kappa Bmt}$ and *ApoE*$^{-/-}$ mice were administered Dox (1.5 mg/ml in drinking water) for 4 days. This Dox treatment regimen is based on our previous time course and dose-response studies [29]. Aortas were harvested from those mice, and the aortic level of I-κBαmt mRNA expression determined using RT-PCR, as we have previously described [29]. The PCR primer set amplifies transgenic I-κBmt, but not endogenous I-κB genes.

2.2. CIH exposure protocol

ApoE$^{-/-}$ and ApoE-EC$^{I\text{-}\kappa Bmt}$ mice were randomly divided into 8 study groups: ApoE$^{-/-}$ sham, ApoE$^{-/-}$ CIH, ApoE$^{-/-}$ HCD, ApoE$^{-/-}$ HCD + CIH, ApoE-EC$^{I\text{-}\kappa Bmt}$-sham, ApoE-EC$^{I\text{-}\kappa Bmt}$-CIH, ApoE-EC$^{I\text{-}\kappa Bmt}$-HCD and ApoE-EC$^{I\text{-}\kappa Bmt}$-HCD + CIH group. To avoid potential gender impact, each study group included equal numbers of males and females. To induce I-κBmt expression, ApoE-EC$^{I\text{-}\kappa Bmt}$ mice were administered Dox in drinking water starting 2 days prior to exposure protocols and continuing during the entire experimental periods. To account for potential Dox effects, *ApoE*$^{-/-}$ mice were treated with Dox in the same manner.

Mice in the sham and CIH groups were fed an NCD (5.0% fat, 0.02% cholesterol, 4 kcal/g) and exposed to sham or CIH starting at 7 weeks of age. Mice in the HCD and HCD + CIH groups were fed an HCD (15.8% fat, 1.25% cholesterol, 4 kcal/g) and exposed to sham or CIH starting at the same age. We placed sham and CIH exposed mice in separate, but identical plexiglass exposure chambers that we have previously described [9,16]. Fractional oxygen concentration in the chamber was reduced to a nadir of 6.0–6.5%, stabilized at that level for 5–7 s, and then gradually increased to 21% over the next 30 s by infusion of nitrogen or air into the chambers by computer controlled solenoid valves. This cycle was repeated every minute over 8 h during the animals' diurnal sleep period. Sham-exposed mice were subjected to similar handling and exposure protocol, but room air was used instead of nitrogen. Our preliminary study showed that long-term exposure to HCD + CIH causes significant mortality. On the other hand, 9 weeks of CIH exposure induces only mild atherosclerotic lesions [9]. Mice in the HCD or HCD + CIH groups were sacrificed after 9 weeks of HCD or HCD + CIH exposure, whereas mice in the sham and CIH groups were sacrificed after 30 weeks of sham or CIH exposure.

2.3. Histological analysis

At completion of exposure protocols, mice were fasted for 8 h, and blood, heart and aorta collected. For *en face* analysis, the aortic tree was perfused with PBS, opened longitudinally, starting from approximately 5 mm distal to the aortic root to the iliac bifurcation. The tissue was fixed and stained with Sudan IV solution, as we previously described [9,16]. To analyze atherosclerotic lesions on aortic roots, the heart was embedded in OCT medium and 6-μm cryostat heart/aortic sections, centered around the aortic valves,