



Chronic intermittent hypoxia induces atherosclerosis by NF- κ B-dependent mechanisms

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ABSTRACT

Chronic intermittent hypoxia (CIH) causes atherosclerosis in mice fed a high cholesterol diet (HCD). The mechanisms by which CIH promotes atherosclerosis are incompletely understood. This study defined the mechanistic role of NF- κ B pathway in CIH + HCD induced atherosclerosis. Wild type (WT) and mice deficient in the p50 subunit of NF- κ B (p50-KO) were fed normal chow diet (ND) or HCD, and exposed to sham or CIH. Atherosclerotic lesions on the *en face* aortic preparation and cross-sections of aortic root were examined. In WT mice, neither CIH nor HCD exposure alone caused, but CIH + HCD caused evident atherosclerotic lesions on both preparations after 20 weeks of exposure. WT mice on ND and exposed to CIH for 35.6 weeks did not develop atherosclerotic lesions. P50 gene deletion diminished CIH + HCD induced NF- κ B activation and abolished CIH + HCD induced atherosclerosis. P50 gene deletion inhibited vascular wall inflammation, reduced hepatic TNF- α level, attenuated the elevation in serum cholesterol level and diminished macrophage foam cell formation induced by CIH + HCD exposure. These results demonstrate that inhibition of NF- κ B activation abrogates the activation of three major atherogenic mechanisms associated with an abolition of CIH + HCD induced atherosclerosis. NF- κ B may be a central common pathway through which CIH + HCD exposure activates multiple atherogenic mechanisms, leading to atherosclerosis.

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1. Introduction

Obstructive sleep apnea (OSA) is one of the most common public health problems [1] and has been recognized as a risk factor for atherosclerotic cardiovascular disease [2–6]. Epidemiological studies have revealed a strong independent association between OSA and coronary [2–6] and cerebral vascular disease [4–7], common consequences of atherosclerosis. However, mechanisms underlying this association remain elusive.

While OSA causes many physiological perturbations, including hypercapnia, arousals, and intrathoracic pressure swings [8,9], long term, repetitive episodes of hypoxia followed by re-oxygenation (chronic intermittent hypoxia, CIH) is believed to be a major contributing factor to

OSA-associated cardiovascular disorders [10]. The focus has therefore been on understanding how CIH causes atherosclerosis.

Several prior studies have demonstrated that 10–12 weeks of CIH exposure induced atherosclerosis in wild type (WT) mice or mice deficient in apolipoprotein E gene (ApoE-KO) on a high cholesterol diet (HCD) [11–14]. Exposure of ApoE-KO mice on normal chow diet (ND) to CIH for 2 weeks exacerbated the progression of pre-existing atherosclerotic lesions [15]. CIH exposure causes hypercholesterolemia and dyslipidemia [11,12,16], systemic and vascular inflammation [17–22], hypertension [10,23] and lipid peroxidation [11–13]. Most recent studies suggest that CIH or OSA impaired triglyceride clearance [24,25]. All of those factors may mediate or contribute to CIH-induced atherosclerosis.

Despite all the evidence, the molecular events orchestrating the activation of multiple atherogenic pathways that lead to atherosclerosis remain largely unexplored. NF- κ B is a multi-faceted transcription factor involved in numerous physiological and pathological processes. In addition to mediating inflammatory and immune responses [26], NF- κ B has been implicated in numerous pathological conditions, including hypertension [27], diabetes [28], obesity [29] and insulin resistance [30], all of which are well-known atherogenic factors. Ryan et al. first demonstrated that intermittent hypoxia/reoxygenation selectively activated NF- κ B in cultured cells [19]. We and others have shown that CIH activated NF- κ B in vascular tissue in mice in an exposure time-dependent manner [17,22]. Patients

Abbreviations: ABCA1, ATP-binding cassette transporter A1; ApoE-KO, mice lacking apolipoprotein E gene; CCL2, chemokine ligand 2; CIH, chronic intermittent hypoxia; EMSA, electrophoretic mobility shift assay; FcO₂, fractional oxygen concentration; HCD, high cholesterol diet; HDL, high density lipoprotein; HMGR, hydroxy-3-methylglutaryl-CoA reductase; IL6, interleukin-6; iNOS, inducible nitric oxide synthase; LDL, low density lipoprotein; LDLr, LDL receptor; ND, normal chow diet; ORO, Oil Red O; OSA, obstructive sleep apnea; p50-KO, mice deficient in NF- κ B p50 gene; SR-B1, scavenger receptor class B1; TRAF3, TNF receptor-associated factor 3

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with OSA had increased NF- κ B activities in circulating neutrophils and monocytes [18] and elevated serum levels of NF- κ B-dependent gene products [18–20]. Thus, the NF- κ B pathway could be a central common pathway through which CIH activates multiple atherogenic mechanisms, leading to atherosclerosis. To test this hypothesis, we inhibited NF- κ B activation using mice deficient in p50 gene (p50-KO). The lack of p50 protein in p50-KO mice diminishes p50/p50 homodimers and reduces nuclear translocation of p50/p65 heterodimer. We examined the effects of NF- κ B inhibition on CIH + HCD-induced atherosclerosis and on the activation of multiple atherogenic mechanisms. We chose to use p50-KO mice, because this mouse strain can tolerate the harsh experimental condition of long term CIH exposure, compared to other NF- κ B mutant mouse strains. We demonstrated that p50 gene deletion diminished CIH + HCD-induced NF- κ B activation, eliminated CIH + HCD induced atherosclerosis, and inhibited CIH + HCD-induced activation of three major atherogenic mechanisms. Our data suggest that activation of NF- κ B pathway plays a central role in CIH + HCD induced atherosclerosis.

2. Materials and methods

2.1. Animal groups and CIH exposure protocol

WT and p50-KO mice were purchased from Jackson laboratory. Animal study protocols were approved by the Feinstein Institute for Medical Research animal care and use committee. We studied 8 groups of mice (all on C57BL/6 genetic background): WT-ND-sham, WT-ND-CIH, WT-HCD-sham, WT-HCD-CIH, p50-KO-ND-sham, p50-KO-ND-CIH, p50-KO-HCD-sham and p50-KO-HCD-CIH groups. Mice were fed ND (5.0% fat, 0.02% cholesterol, 4 kcal/g) or HCD (15.8% fat, 1.25% cholesterol, 4 kcal/g) starting at 7 weeks of age and exposed to sham or CIH.

To mimic the cyclic episodes of hypoxia and reoxygenation seen in OSA patients, we placed sham and CIH exposed mice in separate, but identical plexiglass exposure chambers that we have previously described in detail [17]. The fractional oxygen concentration (F O_2) in the exposure 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 seconds by infusion of nitrogen or air into the chambers by computer controlled solenoid valves. This cycle was repeated every minute over 8 hours during the animals' diurnal sleep period for days according to experimental design. Sham exposed mice were exposed to similar handling but room air was used instead of nitrogen.

To characterize the oxygen profile of our CIH protocol, a cohort of 5 mice was exposed to CIH for 5 days, anesthetized with tribromoethanol, and carotid artery cannulated. Arterial blood was collected when F O_2 in the chamber was at 21% and 6.0–6.5%, respectively, and blood gas analyzed. Another cohort of 5 mice was exposed to CIH for 5 days and Sp O_2 measured using mouse pulse oximeter at day 6.

2.2. Tissue harvest and atherosclerotic lesions analysis

At conclusion of exposure protocols, mice were fasted for 8 hours and sacrificed, and blood, heart, aorta and liver collected. For *en face* analysis of atherosclerotic lesions on aorta, the aortic tree was perfused with PBS, opened longitudinally starting from approximately 5 mm distal to the aortic root to the iliac bifurcation, fixed in a mixture of 5% sucrose and 10% buffered formalin, pinned to a wax pan, and stained with Sudan IV solution [31,32].

For analysis of atherosclerotic lesions on cross section of aortic root, the heart was embedded in OCT medium and serial 6- μ m cryostat heart/aortic sections centered around aortic valves prepared. These sections were stained with Oil Red O (ORO) and hematoxylin as previously described [31,32]. Images of the cross sections and *en face* aortic preparations were captured using a Nikon DS Camera.

The atherosclerotic lesion area was quantified in a blind fashion using Nikon NIS-Elements Research Software and expressed as a percentage of the total area of the entire aorta or as $10^3 \mu\text{m}^2$ on cross section of aortic root.

2.3. Foam cell formation assay

Peritoneal macrophages were isolated by peritoneal lavage of mice 4 days after intraperitoneal injection of thioglycolate medium. Equal numbers of cells from each group were centrifuged onto cytospin slides and stained with ORO and hematoxylin. ORO-positive foam cells were counted and expressed as percentage of total cell counts.

2.4. Electrophoretic mobility shift assay (EMSA)

Nuclear and cytoplasmic proteins were extracted from liver, and NF- κ B binding activity was measured by EMSA using ^{32}P -labeled NF- κ B consensus oligonucleotide as we have previously described [33]. The specificity of NF- κ B DNA binding was confirmed in competition reactions in which 50-fold molar excess unlabelled NF- κ B oligonucleotide was added to the binding reaction 10 minutes prior to the addition of labeled probe.

2.5. Western blotting

Equal amounts of proteins were separated on 7.5–10% SDS-PAGE slab gel under denaturing conditions. Tissue levels of TNF receptor-associated factor 3 (TRAF3), p100, I- κ B α , ATP-binding cassette transporter A1 (ABCA1), scavenger receptor class B1 (SR-B1) and actin were detected by Western blotting as we previously described [34].

2.6. Measurement of serum levels of lipids, lipoproteins and cytokines

Serum levels of total cholesterol, HDL and LDL/VLDL cholesterol and triglycerides were measured using commercial kits (BioAssay, Pointe Scientific). Serum levels of interleukin-6 (IL6), chemokine ligand (CCL) 2 and TNF- α , and hepatic level of TNF- α were measured using ELISA kits (eBiosciences).

2.7. Immunohistochemical staining

Cryosections (6 μm) were prepared, fixed, permeabilized, blocked in blocking solution, and incubated with anti-iNOS (inducible nitric oxide synthase) or anti-Mac3 antibodies (Santa Cruz) as we previously described [34]. Specific binding was detected with biotinylated secondary antibody-horseradish peroxidase complexes using VECTASTAIN® Elite ABC kits (Vector Laboratories). Antigen-antibody complexes were visualized using 3', 3'-diamino benzidine (Vector Laboratories). Sections were counterstained with hematoxylin, mounted and viewed under light microscope. Total and iNOS (or MAC3) positive areas were quantified in 5 random microscopic fields per aortic section under the same magnification. The accumulated iNOS (or MAC3) positive area from the 5 microscopic fields was divided by the accumulated total analyzed area of aortic wall.

2.8. Q-RT-PCR

Total RNA was extracted using TRIZOL reagent (Invitrogen, CA) and treated with RNase-free DNase I to remove traces of genomic DNA. Each RNA sample was subjected to quantitative reverse transcription and polymerase chain reaction (Q-RT-PCR) using TaqMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems) and gene specific primers and probes. The gene specific probes were: #78 for hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR), #64 for LDL receptor (LDLr), #1 for ABCA1 and #2 for SR-B1 (Roche Universal Probe Library, Mannheim, Germany). Primers used were: HMGCR, forward, 5'-tgattgg

Table 1
Mean body weight and serum lipid and lipoprotein levels.

	N	Body weight (g)	Triglyceride (mg/dl)	Cholesterol (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)
WT-ND-sham	8	27.6 ± 1.8	39.1 ± 3.2	48.5 ± 4.0	53.3 ± 7.0	22.5 ± 4.5
WT-HCD-sham	8	24.3 ± 1.0	73.8 ± 40.4	166.7 ± 17.2*	51.8 ± 5.0	71.9 ± 9.7#
WT-ND-CIH	8	23.6 ± 0.7	43.2 ± 7.5	44.4 ± 6.3	50.8 ± 10.7	24.9 ± 5.4
WT-HCD-CIH	8	25.1 ± 1.1	84.3 ± 45.4	176.6 ± 20.4*	49.7 ± 6.4	83.2 ± 15.3#
p50-KO-ND-sham	6	29.0 ± 1.8	35.3 ± 10.2	62.8 ± 4.9	70.7 ± 5.4	22.3 ± 1.9
p50-KO-HCD-sham	6	26.7 ± 1.6	20.0 ± 3.7	145.6 ± 13.4*	51.1 ± 4.6	59.1 ± 7.6#
p50-KO-ND-CIH	6	23.6 ± 0.6*	27.3 ± 3.0	56.5 ± 3.9	58.1 ± 8.7	16.2 ± 1.7
p50-KO-HCD-CIH	6	21.4 ± 1.0*	23.9 ± 7.1	110.5 ± 10*‡	27.8 ± 4.4#‡	77.8 ± 25.3#

*, significantly different from their respective ND-Sham group. #, significantly different from their respective ND-Sham and ND-CIH groups. ‡, significantly different from WT-HCD-CIH group.

agttggcaccat-3' and reverse, 5'-tggccaacactgacatgc-3'; LDL receptor, forward, 5'-gatggctatactaccctcaa-3' and reverse, 5'-tgctcatgccaca tcgtc-3', ABCA1, forward, 5'-gcagatcaagcatcccaact-3' and reverse, 5'-cc agagaatgtttcattgtcca; SR-B1, forward, 5'-gccatcatctgccaaact-3' and

reverse, 5'-tcctggggagcccttttact-3'. Q-RT-PCR Was performed in 7900 HT thermocycler (Applied Biosystems). Results were analyzed using the delta-delta C_t method. Expression of specific genes was normalized to β-actin mRNA (TaqMan RNA Reagents, Applied Biosystems).

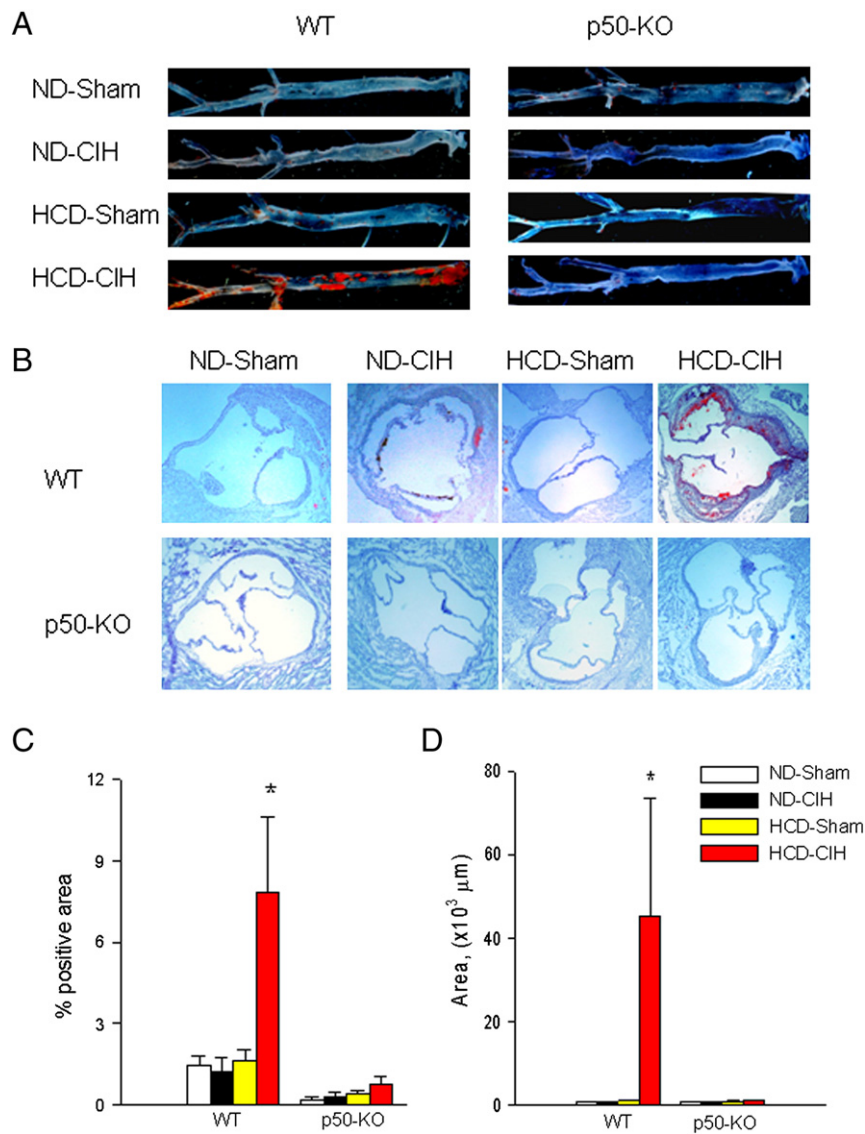


Fig. 1. P50 gene deletion abolishes CIH + HCD induced atherosclerosis. Wild type (WT) mice and mice deficient in p50 gene (p50-KO) were fed normal chow diet (ND) or high cholesterol diet (HCD) starting at 7 weeks of age, and exposed to sham (ND-Sham and HCD-Sham) or CIH (ND-CIH and HCD-CIH). At 20 weeks after sham or CIH exposure, the *en face* aortic preparations and cross-sections of aortic root were prepared and stained with Sudan IV and Oil Red O (ORO), respectively. **A:** Representative photographs of *en face* aortic preparations showing that Sudan IV-stained atherosclerotic lesions were detected only on aorta from WT-HCD-CIH group of mice. **B:** Representative photographs of cross-sections of aortic root showing that ORO-stained atherosclerotic lesions were seen only on section from WT-HCD-CIH group of mice. **C:** Atherosclerotic lesion area on *en face* aortic preparation was quantified and expressed as a percentage of total aortic area. Means ± SEM of 8 animals in each group. **p* < 0.05, compared with other 7 groups. **D:** Atherosclerotic lesion area on cross-sections of aortic root was quantified and expressed as × 10³ μm. Means ± SEM of 8 animals in each group. **p* < 0.05, compared with other 7 groups.

2.9. Statistical analysis

Data were expressed as mean \pm SEM, and analyzed using unpaired *t* test or Mann–Whitney rank test for comparing two groups. For comparing multiple groups, data were analyzed using ANOVA or Kruskal–Wallis rank test, followed by Holm–Sidak method or Student–Newman–Keuls Method for *post hoc* analysis. The null hypothesis was rejected at 5% level.

3. Results

3.1. Characterization of the mouse model of CIH

Body weights were comparable among the 8 groups at the commencement of the exposure protocols. At the completion of the exposure protocols (20 weeks), mice in experimental groups had lower body weights, compared to respective ND-sham groups. Statistically significant differences were noted between ND-sham and ND-CIH or ND-sham and HCD + CIH groups of p50-KO mice (Table 1).

Blood gas analysis on a group of anesthetized mice and mouse pulse oximeter measurement on another group of awake mice showed that a decline in fractional oxygen concentration (F_O₂) from 20.9% to a nadir of 6% over 30-s hypoxic period resulted in a drop in PaO₂ from 96.5 \pm

3.8 to 45.8 \pm 1.7 mm Hg, and a drop in pulse oximeter oxygen saturation (SpO₂) from 97.7 \pm 0.3 to 79.3 \pm 1.2%. Our result is consistent with a report showing that PaO₂ dropped from 88 \pm 3 at room air to 47 \pm 2 mm Hg at the nadir of 6% O₂ in conscious mice [35]. Our mouse model produces a level of hypoxia that resembles moderate to severe OSA, and a rate of hypoxia (60 events/hour) which simulates severe OSA [8,9].

3.2. Effects of CIH exposure on the development of atherosclerosis in WT mice

A prior study showed that C57 WT mice on ND had no detectable atherosclerotic lesions after 12 weeks of CIH exposure [11]. It is possible that exposure to CIH for longer periods of time may cause atherosclerosis. To examine this possibility, we exposed WT mice on ND to sham or CIH for 20 weeks. After 20 weeks of CIH exposure, atherosclerotic lesions were not detected on *en face* aortic

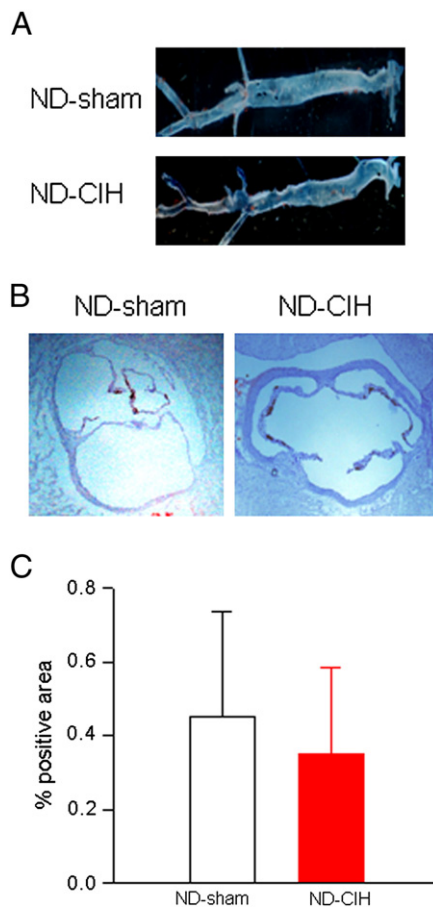


Fig. 2. Long-term CIH exposure does not cause atherosclerosis. WT mice were fed ND and exposed to sham (ND-Sham) or CIH (ND-CIH) for 35.6 weeks. *En face* aortic preparations and cross-sections of aortic root were prepared and stained with Sudan IV and ORO, respectively. **A** and **B**: Representative photographs of *en face* aortic preparations and cross-sections of aortic root. Sudan IV- or ORO-positive atherosclerotic lesion was hardly detected following 8.3 months of CIH exposure. **C**: Atherosclerotic lesion area on *en face* aortic preparation was quantified and expressed as a percentage of total aortic area. Means \pm of 5 animals in each group.

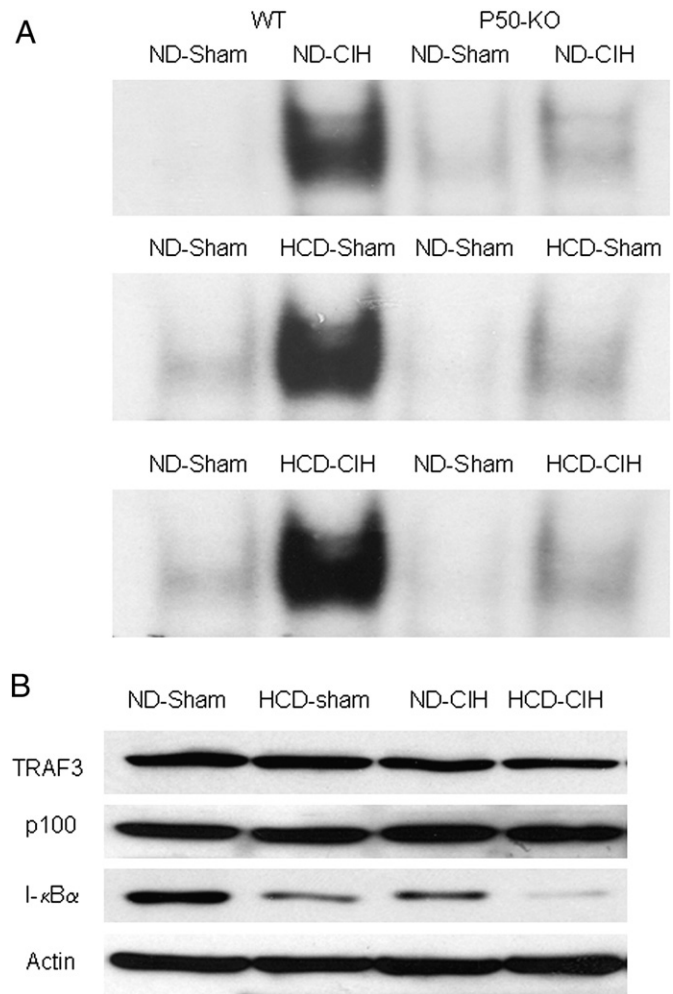


Fig. 3. P50 gene deletion blocks CIH-, HCD- or CIH + HCD-induced NF-κB activation. Mice were fed ND or HCD and exposed to sham (ND-Sham, HCD-Sham) or CIH (ND-CIH, HCD-CIH) for 30 days. Hepatic NF-κB DNA binding activity was measured by electrophoretic mobility shift assay (EMSA) and hepatic levels of TNF receptor associate factor 3 (TRAF3), p100/NF-κB2, I-κBα and actin proteins were determined by Western blot. **A**: EMSA autoradiograph showing that p50 gene deletion blocks CIH (top), HCD (middle) or CIH + HCD (bottom) induced NF-κB activation. **B**: Characterization of CIH-, HCD- or CIH + HCD-induced NF-κB activation pathway. Western blot photographs showing hepatic levels of TRAF3, p100, I-κBα and actin proteins. CIH, HCD or CIH + HCD did not cause TRAF3 and p100 degradation, but significantly reduced tissue level of I-κBα, illustrating that CIH, HCD and CIH + HCD activate NF-κB via the canonical pathway.

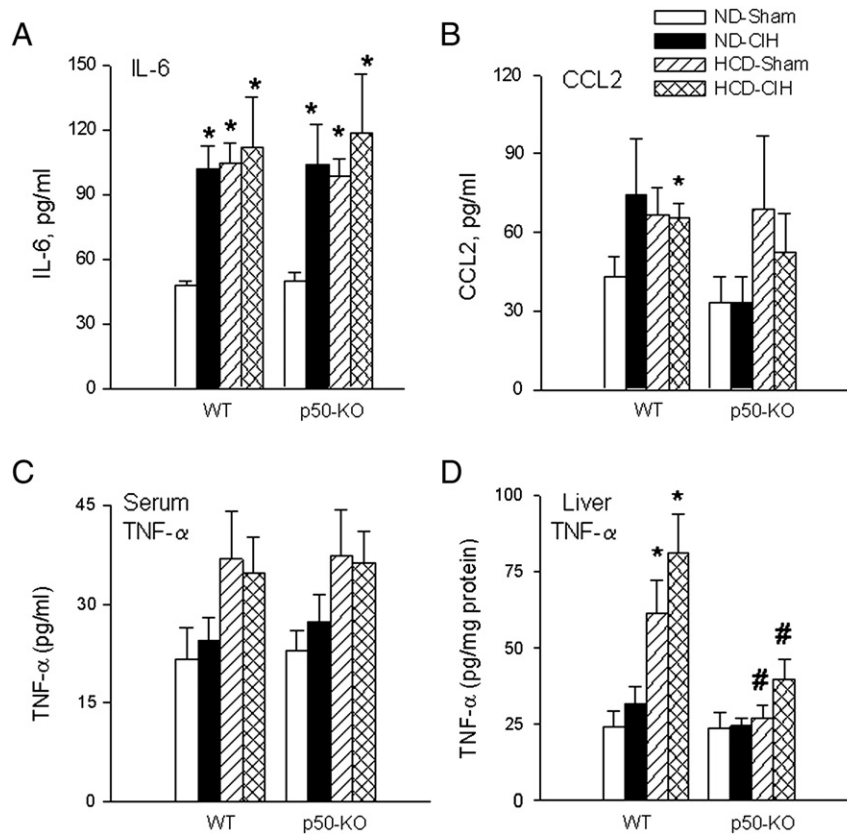


Fig. 4. Effects of CIH and p50 gene deletion on serum levels of markers of systemic inflammation and hepatic level of TNF- α . Mice on ND or HCD were exposed to sham (ND-Sham, HCD-Sham) or CIH (ND-CIH, HCD-CIH) for 9 weeks. Serum levels of IL-6 (A), CCL2 (B) and TNF- α (C), and hepatic level of TNF- α (D) were determined using ELISA kit. CIH and/or HCD exposure increased serum level of IL-6, but not TNF- α . CIH + HCD and p50 gene deletion had different effects on serum and hepatic TNF- α . Means \pm SEM of 6 mice each group. * p < 0.05, compared to respective ND-Sham group. # p < 0.05, compared to WT-HCD-Sham or WT-HCD-CIH group.

preparations and on cross-sections of aortic root (Fig. 1A and B, WT, ND-sham vs ND-CIH). To further clarify that CIH exposure does not cause atherosclerosis in WT mice, we exposed another cohort of WT mice on ND to CIH for 35.6 weeks. The long-term exposure to CIH did not cause atherosclerotic lesions (Fig. 2).

Next, we examined if HCD alone causes atherosclerosis. Atherosclerotic lesions were not detectable on *en face* aortic preparations or cross-sections of aortic root from WT mice fed HCD for 20 weeks (Fig. 1A and B, WT, ND-sham vs HCD-sham). By contrast, atherosclerotic lesions were detected on both preparations from WT-HCD-CIH group of mice (Fig. 1A and B, WT, ND-sham vs HCD-CIH). Fig. 1C and D (left panel) showed quantification of atherosclerotic lesion areas on *en face* aortic preparations and on the cross-sections of aortic root. Thus, neither CIH nor HCD alone causes atherosclerosis, but a combination of the two causes pronounced atherosclerotic lesions in C57BL WT mice.

3.3. P50 gene deletion abolishes CIH + HCD-induced atherosclerosis

To define the role of NF- κ B activation in CIH + HCD-induced atherosclerosis, we compared atherosclerotic lesion areas between WT and p50-KO mice. After 20 weeks of CIH exposure, atherosclerotic lesions were detected on the *en face* aortic preparations and cross-sections of aortic root from WT-HCD-CIH group (Fig. 1A and B, WT, HCD-CIH). The CIH + HCD-induced atherosclerosis was abolished in p50-KO-HCD-CIH group of mice (Fig. 1A and B, HCD-CIH, WT vs p50-KO), indicating that NF- κ B activation plays a pivotal role in CIH + HCD-induced atherosclerosis.

CIH-induced NF- κ B activation, vascular inflammation and dyslipidemia exhibited different time course profiles. NF- κ B

activation and vascular inflammation peaked at 4–5 weeks and 8–10 weeks, respectively. To demonstrate an effect of NF- κ B inhibition, measurement of each response has to be timed to catch the response at its peak. Each response was studied at different time point in subsequent studies.

3.4. P50 gene deletion blocks CIH- and/or HCD-induced NF- κ B activation

To examine the mechanisms by which p50 KO abolishes CIH + HCD-induced atherosclerosis, we first wanted to confirm if p50 gene deletion blocks CIH + HCD-induced NF- κ B activation. A preliminary study showed that the time course profiles of CIH-induced NF- κ B activation and the subunit compositions of CIH-induced NF- κ B complex are identical between aorta and liver. We analyzed NF- κ B activation pathway using hepatic proteins to save aortas for more important analyses, such as atherosclerotic lesion analyses. Liver is a major target of CIH exposure and alteration in liver gene expression plays important roles in CIH-induced dyslipidemia and atherosclerosis [11,12,16]. Compared to ND-sham group, ND-CIH, HCD-sham or HCD-CIH group of WT mice showed a markedly increased hepatic NF- κ B activity, which was significantly inhibited in all the three groups of p50-KO mice (Fig. 3A), demonstrating that p50 gene deletion blocks CIH- and/or HCD-induced NF- κ B activation.

Western blot analysis revealed that CIH, HCD or CIH + HCD exposure had no effects on hepatic levels of TRAF3 and p100 proteins (Fig. 3B, TRAF3 and p100), two key signaling molecules of the noncanonical NF- κ B activation pathway [26,36], but significantly reduced hepatic level of I- κ B α protein, key signaling molecule of the canonical NF- κ B

activation pathway [26,36] (Fig. 3B, I- κ B α). Thus, CIH and/or HCD activate NF- κ B via the canonical pathway in WT C57BL/6 mice.

3.5. P50 gene deletion inhibits CIH + HCD-induced vascular wall inflammation

We compared serum levels of TNF- α , IL6 and chemokine ligand (CCL)₂, and aortic iNOS expression between WT and p50-KO mice. CCL₂, also known as monocyte chemoattractant protein-1, mediates monocyte recruitment into developing atherosclerotic lesions. We chose to study those NF- κ B-regulated gene products because of their established roles in atherogenesis [37–40]. CIH, HCD or HCD + CIH exposure all significantly increased serum level of IL6 (Fig. 4A). HCD + CIH, but not CIH or HCD alone, significantly increased serum level of CCL₂ (Fig. 4B). P50 gene deletion had no significant effects on CIH and/or HCD induced changes in serum levels of the 2 cytokines (Fig. 4A and B). CIH/HCD and p50 gene deletion had different effects on serum and hepatic levels of TNF- α . HCD or HCD + CIH exposure did not significantly increase serum level (Fig. 4C), but significantly increased hepatic level of TNF- α (Fig. 4D), which was inhibited by p50 gene deletion (Fig. 4D). Thus, CIH and/or HCD exposure had variable effects on serum levels of markers of systemic inflammation and p50 gene deletion did not alter the effects.

We next examined if p50 KO affects CIH- and/or HCD-induced vascular wall inflammation. IHC staining showed that CIH or HCD increased, and CIH + HCD further increased aortic iNOS expression (Fig. 5, WT, ND-sham vs ND-CIH, HCD-sham or HCD + CIH). P50 gene deletion significantly inhibited iNOS expression caused by each

stimulus (Fig. 5). Likewise, CIH or HCD significantly increased and CIH + HCD further increased aortic infiltration of Mac3-positive macrophages (Fig. 6, WT, ND-sham vs ND-CIH, HCD-sham or HCD + CIH), which was attenuated by p50 gene deletion (Fig. 6). Thus, p50 gene deletion inhibits vascular wall inflammation.

3.6. P50 gene deletion attenuates CIH + HCD-induced hypercholesterolemia

Serum levels of lipids and lipoproteins in the 8 groups of mice were summarized in Table 1. HCD alone or CIH + HCD, but not CIH alone, significantly increased serum levels of total cholesterol and LDL-cholesterol in WT mice. P50 gene deletion significantly reduced HCD + CIH-induced, but not HCD-induced, serum level of total cholesterol. P50 gene deletion had no significant effect on HCD- or CIH + HCD-induced changes in serum LDL-cholesterol level (Table 1).

3.7. P50 gene deletion prevents SR-B1 and ABCA1 down-regulation

QRT-PCR analysis revealed that CIH + HCD significantly down-regulated hepatic ABCA1 and SR-B1 mRNA expressions, which were prevented by p50 gene deletion (Fig. 7A and B). Consistent with the changes in mRNA levels, hepatic ABCA1 and SR-B1 protein expressions were down-regulated by CIH + HCD in WT, but not in p50-KO, mice (Fig. 7E, WT vs p50-KO). CIH + HCD and p50 gene deletion had no significant effects on hepatic HMGCR and LDLr mRNA expressions (Fig. 7C and D).

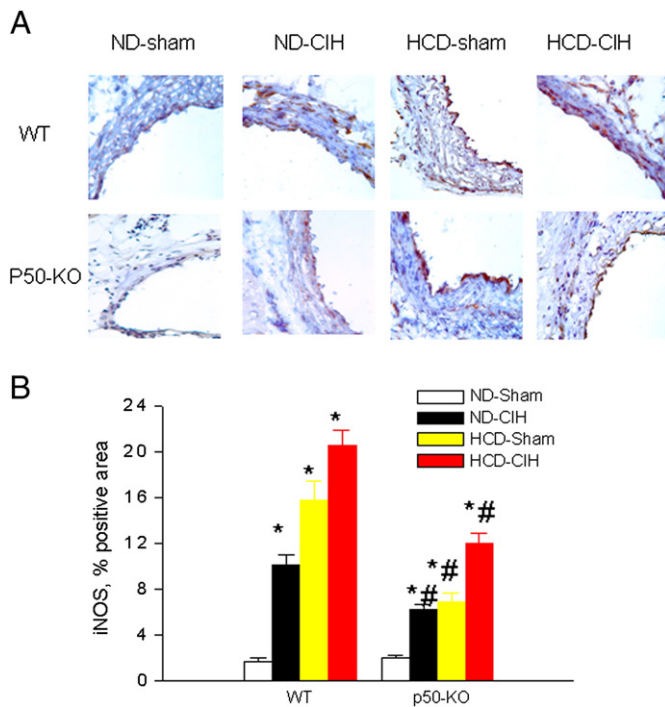


Fig. 5. P50 gene deletion inhibits CIH-, HCD- or CIH + HCD-induced aortic iNOS expression. Mice were fed ND or HCD and exposed to sham (ND-Sham, HCD-Sham) or CIH (ND-CIH, HCD + CIH) for 9 weeks. Aortic iNOS protein expression was detected by IHC staining using iNOS specific antibody. **A:** IHC staining photographs showing that CIH (WT, ND-sham vs ND-CIH), HCD (WT, ND-sham vs HCD-sham) and HCD + CIH (WT, ND-sham vs HCD + CIH) increased aortic iNOS expression, and that p50 gene deletion inhibited CIH- (ND-CIH, WT vs p50-KO), HCD- (HCD-sham, WT vs p50-KO) and HCD + CIH-induced (HCD + CIH, WT vs p50-KO) aortic iNOS expression. **B:** Quantitative analysis of the iNOS-positive area expressed as a percentage of total analyzed area. Means \pm SEM of 5 mice in each group. * p < 0.05, compared to respective ND-sham group. # p < 0.05, compared to WT-ND-CIH, WT-HCD-sham or WT-HCD-CIH group.

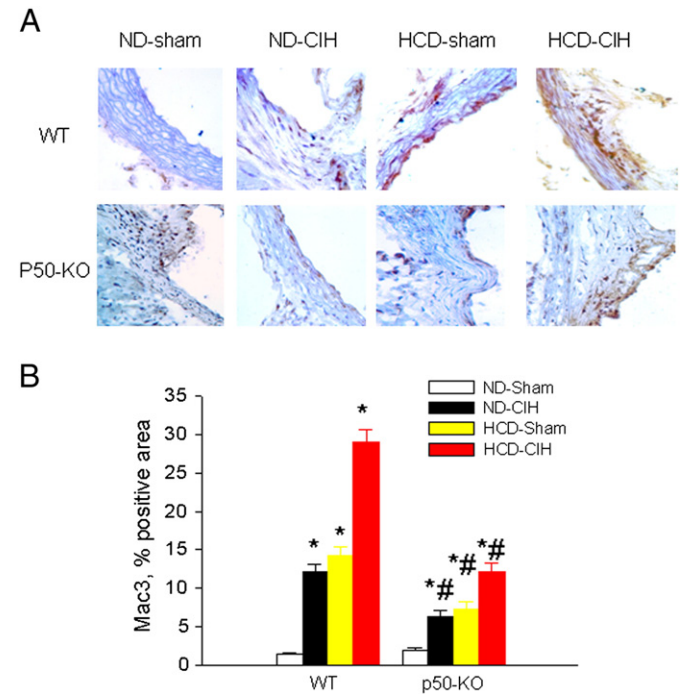


Fig. 6. P50 gene deletion inhibits CIH-, HCD- or CIH + HCD-induced aortic macrophage infiltration. Mice were fed ND or HCD and exposed to sham (ND-Sham, HCD-Sham) or CIH (ND-CIH, HCD + CIH) for 9 weeks. Aortic macrophage infiltration was detected by IHC staining using Mac3 specific antibody. **A:** IHC staining photographs showing that CIH (WT, ND-sham vs ND-CIH), HCD (WT, ND-sham vs HCD-sham) and HCD + CIH (WT, ND-sham vs HCD + CIH) increased aortic macrophage infiltration, and that p50 gene deletion inhibited CIH- (ND-CIH, WT vs p50-KO), HCD- (HCD-sham, WT vs p50-KO) and HCD + CIH-induced (HCD + CIH, WT vs p50-KO) aortic macrophage infiltration. **B:** Quantitative analysis of the Mac3-positive area expressed as a percentage of total analyzed area. Means \pm SEM of 5 mice in each group. * p < 0.05, compared to respective ND-sham group. # p < 0.05, compared to WT-ND-CIH, WT-HCD-sham or WT-HCD-CIH group.

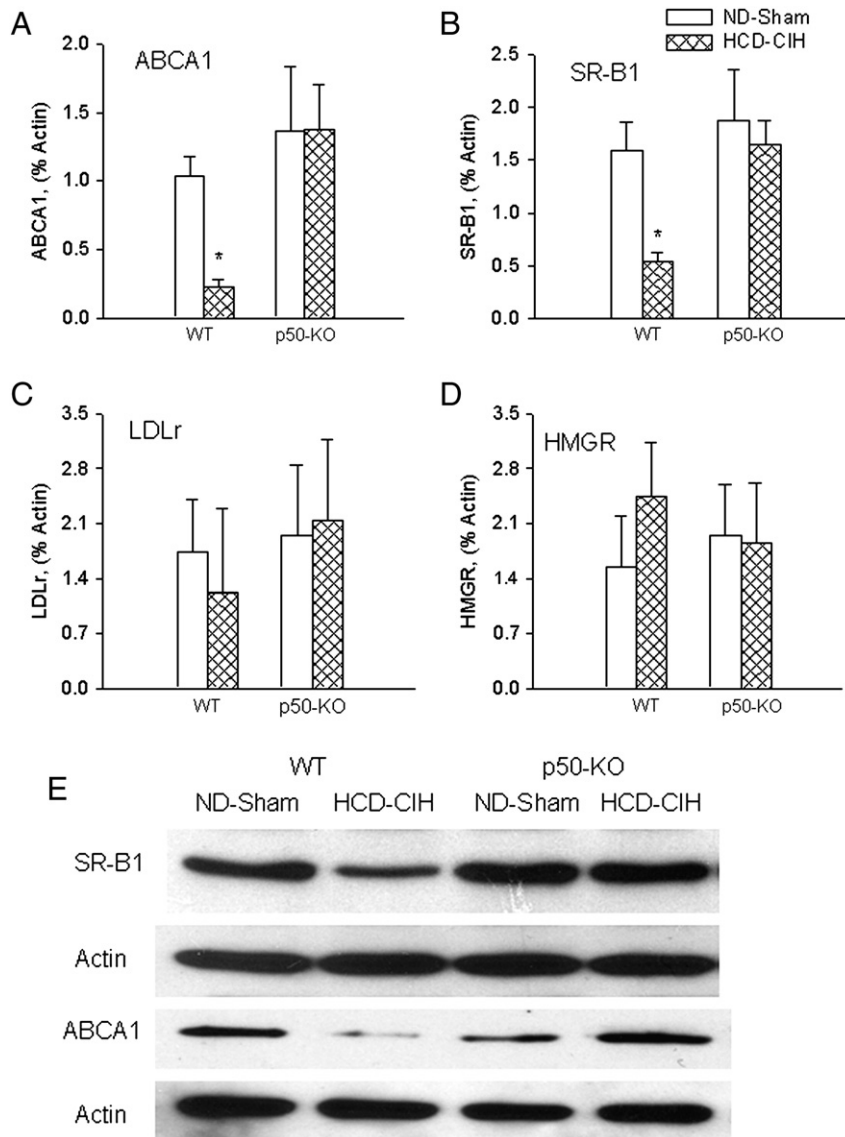


Fig. 7. P50 gene deletion prevents CIH + HCD-induced down-regulation of ABCA1 and SR-B1 expression. Mice on ND or HCD were exposed to sham (ND-Sham) or CIH (HCD + CIH) for 9 weeks, and liver collected. Hepatic levels of ATP-binding cassette transporter A1 (ABCA1), scavenger receptor class B1 (SR-B1), LDL receptor (LDLr) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) mRNAs expressions were quantified using Q-RT-PCR, and normalized to level of β -actin mRNA. Hepatic levels of ABCA1 and SR-B1 proteins were determined by Western blot. **A–D:** P50 gene deletion prevents CIH + HCD-induced ABCA1 and SR-B1 mRNA down-regulation. Mean \pm SEM of 5 mice. * $P < 0.05$ compared with other 3 groups. **E:** Western blot photographs showing that p50 gene deletion prevents CIH + HCD-induced hepatic ABCA1 and SR-B1 protein down-regulation. Actin: membrane for ABCA1 or SR-B1 blotting was re-blotting to actin antibody.

3.8. P50 gene deletion reduces CIH + HCD-induced foam cells

Neither CIH nor HCD exposure alone caused macrophage foam cell formation, but CIH + HCD induced significant number of macrophage foam cells in WT mice (Fig. 8). The CIH + HCD-induced foam cell formation was diminished in p50-KO mice (Fig. 8), indicating a pivotal role for NF- κ B in CIH + HCD induced macrophage foam cell formation.

4. Discussion

A major finding of this study is that activation of NF- κ B pathway plays a central role in CIH + HCD-induced atherosclerosis. Deletion of p50 gene diminished CIH + HCD induced NF- κ B activity and concurrently eliminated CIH + HCD induced atherosclerosis in C57BL/6 mice. P50 gene deletion inhibited the activation of three major atherogenic mechanisms, vascular inflammation, hypercholesterolemia and macrophage foam cell formation. Thus, NF- κ B pathway may be a central

common pathway through which CIH activates multiple atherogenic mechanisms that act synergistically to induce atherosclerosis.

NF- κ B activation is driving force of inflammation [26]. Inflammation participates in all stages of the classic atherosclerosis [41]. CIH activates NF- κ B and NF- κ B-mediated inflammatory pathways [17–22]. However, the causative role of NF- κ B activation in CIH-induced atherosclerosis has not been previously studied. We demonstrated here that CIH and HCD activated NF- κ B, and synergistically caused vascular inflammation and atherosclerosis. Deletion of p50 gene diminished NF- κ B activity, inhibited vascular inflammation, and abolished atherosclerotic lesions induced by CIH + HCD. These data demonstrate a causal role of NF- κ B activation in CIH + HCD-induced atherosclerosis.

P50 gene deletion did not significantly reduce CIH + HCD-induced serum levels of markers of systemic inflammation, despite abolishing atherosclerosis. P50 gene deletion significantly inhibited CIH + HCD-induced vascular wall inflammation. These results suggest that vascular inflammation may be a more relevant factor for CIH-evoked atherosclerosis,

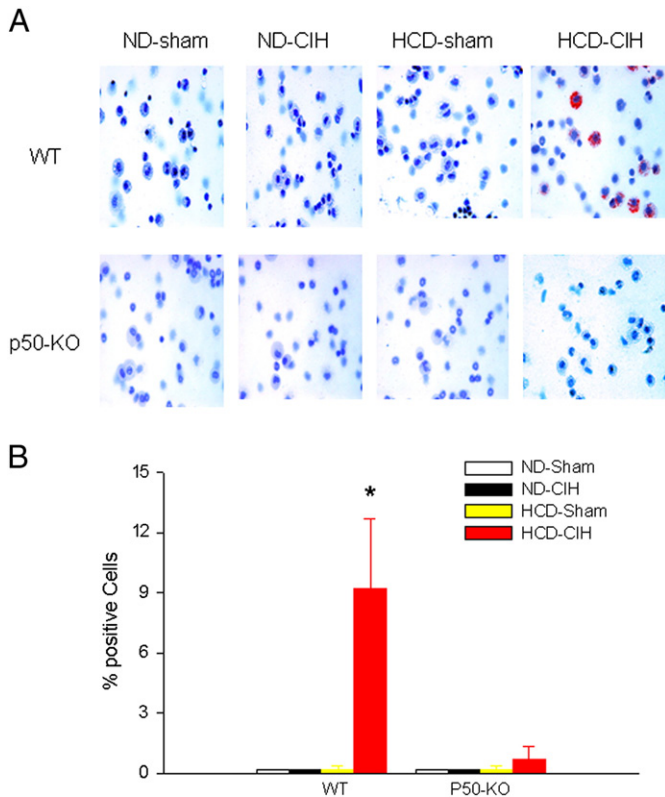


Fig. 8. P50 gene deletion inhibits CIH + HCD-induced macrophage foam cell formation. Mice on ND or HCD were exposed to sham (ND-Sham, HCD-Sham) or CIH (ND-CIH, HCD-CIH) for 9 weeks. Peritoneal macrophages were harvested, centrifuged onto cytopsin slides and stained with ORO and hematoxylin. **A:** Representative photographs showing that p50 gene deletion inhibited CIH + HCD-induced macrophage foam cell formation. **B:** ORO-positive foam cells were counted and expressed as percentage of total cell counts. Means \pm SEM of 5 mice in each group. * $p < 0.05$, compared to other 7 groups.

although systemic inflammation can be an additional factor. It is noteworthy that CIH may have different effects on systemic and local inflammation. A previous report showed that CIH + HCD significantly increased hepatic, but not serum level of TNF- α [11]. Different effects of CIH + HCD on serum and hepatic TNF- α levels were also observed in the current study. More importantly, we found that p50 gene deletion significantly reduced hepatic, but not serum TNF- α level. The discrepant effects of CIH + HCD and p50 gene deletion on systemic and liver inflammation may be related to cell type-dependent variations in sensitivity and/or response to CIH and to NF- κ B inhibition. This speculation is supported by our previous study showing that vascular tissue is more sensitive than other tissues to CIH in term of NF- κ B activation [17].

P50 gene deletion inhibited both CIH- and HCD-induced vascular inflammation, suggesting a non-specific anti-inflammatory effect. However, this cannot rule out the possibility that CIH and HCD may activate NF- κ B pathway through distinct mechanisms or signaling pathways. Further studies are needed to elucidate these mechanisms and pathways.

Hypercholesterolemia is a major mechanism of atherosclerosis [42]. Our demonstration that p50 gene deletion significantly reduced CIH + HCD-induced serum cholesterol level and concomitantly abolished CIH + HCD-induced atherosclerosis indicates that NF- κ B activation mediates, at least partially, the atherogenic response to CIH + HCD by causing hypercholesterolemia. To elucidate the mechanistic link between NF- κ B activation and hypercholesterolemia, we examined the effects of p50 gene deletion on CIH + HCD-induced changes in the expressions of HMGCR, the rate-limiting enzyme in cholesterol biosynthesis, LDLr, SR-B1 and ABCA1, receptors mediating cholesterol uptake and clearance. Neither CIH + HCD exposure nor

p50 gene deletion affected HMGCR and LDLr mRNA expression. CIH + HCD down-regulated ABCA1 and SR-B1 mRNA and protein expressions and p50 gene deletion prevented the down-regulation, indicating a role for NF- κ B in the down-regulation. Previous studies have shown that CIH exposure down-regulated SR-B1 protein expression [16], and that oxidized LDL and cytokine down-regulated ABCA1 expression in an NF- κ B-dependent manner [43]. Collectively, these results support the contention that CIH activates NF- κ B, which down-regulates SR-B1 and ABCA1 expression, resulting in a perturbation in cholesterol clearance and hypercholesterolemia.

We demonstrated for the first time that CIH exposure promotes macrophage foam cell formation in WT mice on a HCD. An important role for NF- κ B in the CIH + HCD-induced foam cell formation is indicated by our demonstration that CIH + HCD induced significant numbers of macrophage foam cells in WT, but not in p50-KO mice. Our result is consistent with a previous report showing that macrophage-specific overexpression of a mutant I- κ B α suppressed HCD-induced macrophage foam cell formation [44]. NF- κ B activation could stimulate foam cell formation by several mechanisms. NF- κ B target gene products, including TNF- α , IL-1 β and IFN- γ up-regulate acylcholesterol transferase (ACAT) expression [45,46] and/or down-regulate the expression of scavenger receptors [43,44]. NF- κ B mediates the up-regulation of ACAT [45] and down-regulation of scavenger receptors [43]. Altered expression of ACAT and scavenger receptors increases cholesterol uptake, decreases cholesterol efflux and increases macrophage intracellular cholesterol storage, stimulating foam cell formation.

The pathogenic role of macrophage foam cell formation in atherosclerosis is well documented [47]. CIH and HCD synergistically induced macrophage foam cells and atherosclerosis. P50 gene deletion diminished CIH + HCD-induced foam cell formation and atherosclerosis. It is possible that CIH + HCD activate NF- κ B, which in turn promotes atherosclerosis by inducing macrophage foam cell formation.

NF- κ B activation is a pathogenic mechanism of hypertension [27], diabetes [28], obesity [29] and insulin resistance [30], conditions known to cause atherosclerosis and to be associated with CIH exposure and OSA. NF- κ B activation by CIH could promote atherosclerosis by inducing these conditions. Overall, these results support our hypothesis that NF- κ B activation serves as a central common mechanism by which CIH activates multiple atherogenic mechanisms, leading to atherosclerosis.

A prior study showed that exposure of WT mice on ND to CIH for 12 weeks did not cause atherosclerosis [11]. However, exposure to CIH for longer periods of time may cause atherosclerosis. We exposed WT mice on ND to CIH for up to 8.3 months and found no evidence of atherosclerosis. Our study therefore extends previous studies [11–14] by definitely approving that CIH exposure alone does not cause atherosclerosis in WT C57BL/6 mice.

Although this is an animal study, the concept demonstrated here could be applicable to OSA patients. Patients with OSA have increased NF- κ B activity in neutrophils and monocytes [18] and increased plasma levels of NF- κ B-regulated gene products [18–20]. It is possible that OSA, by causing CIH, activates NF- κ B, which in turn activates multiple atherogenic mechanisms, leading to a proatherogenic phenotype. The important role of NF- κ B in CIH-induced atherosclerosis suggests that NF- κ B may be a good target for developing therapies. Targeting NF- κ B pathway can simultaneously inhibit multiple atherogenic mechanisms and will be very effective. One major caveat for targeting NF- κ B is that it causes unwanted side effects, because NF- κ B is involved in many cellular and physiological processes. A strong argument for targeting the NF- κ B pathway is that a vast majority, if not all, of signaling molecules that play important physiological roles are multifaceted. To maximize therapeutic effects and minimize the side effects, one useful strategy would be to block the NF- κ B pathway in a cell-targeted and pathological stage-specific manner. With the advance of modern drug delivery technology, it is quite feasible to deliver small molecule NF- κ B inhibitors to targeted cells at a specific disease stage.

One limitation of this study was that CIH/HCD exposure caused weight loss, especially in p50-KO mice. Since food restriction induced weight loss correlated with reduced atherosclerosis in obese and insulin-resistant mice [48], the possibility that CIH/HCD-induced weight loss may have partially contributed to the protection against atherosclerosis in p50-KO mice cannot be ruled out. However, NF- κ B-mediated activation of multiple atherogenic mechanisms clearly plays an important role.

Author contributions

S.F.L. developed the concept, designed the experiments and prepared the manuscript. Y.G. participated in experiments design. D.S. and G.F. performed majority of the biochemical, histological and IHC experiments. S-Z.M. and X.Y. performed parts of the biochemical and histological experiments and participated in animal exposure protocols. G.L. participated in animal exposure protocols and tissue collection.

Conflict of interest statement

The authors declare no conflict of interest.

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