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Prenatal hypoxia promotes atherosclerosis via vascular inflammation in the offspring rats

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ABSTRACT

Background: Hypoxia is a critical contributor to increased risks of cardiovascular diseases, including atherosclerosis, but the detailed mechanism that hypoxia leads to atherosclerosis remains unknown.
Methods: Pregnant rats were treated with hypoxia (10.5% oxygen) during pregnancy, and HUVEC cells treated with 1% of oxygen. Blood lipids were tested at fetal stage and adult stage of offspring rats; the level of pro-inflammatory cytokines of HUVEC and offspring rats were investigated, and HIF-1α and NFκB mRNA level were also measured by Q-PCR and Elisa.
Results: We found that TC, LDL-C, ox-LDL-C, and the receptors of ox-LDL-C (lox-1) of the adult offspring were significantly higher than that of the control, while HDL-C was significantly reduced in hypoxia group. The internal elastic lamina was blocked by smooth muscle cells; and the migration of smooth muscle cells into the intima were observed in hypoxia offspring. Luciferase reporter gene experiment showed that HIF-1α activated NFκB transcription at four discrete binding sites of NFκBp65 promoter, although there was no obvious difference among the four discrete binding sites. Using transfection of pCDNA3.1-HIF-1α on HUVEC cells, HIF-1α significantly activated NFκB transcription at hypoxic conditions (1% O_2), and concurrent with increased expression of IL-1β and TNF-α.
Conclusion: Hypoxia during pregnancy activated NFκB transcription to induce pro-inflammatory cytokines, leading to the early stage of atherosclerosis.

1. Introduction

Recent studies indicate that infants are at an increased risk of cardiovascular diseases and atherosclerosis in late life as a result of fetal intrauterine growth restriction (IUGR). One of the most important factors leading to IUGR is hypoxia [1,2]. However, the impact of prenatal hypoxia during pregnancy on the development of atherosclerosis in the offspring is still unclear.

Hypoxia may cause vascular damage, including intima thickening, endothelial dysfunction, proliferation and migration of media smooth muscle cells to intima, inflammatory cells infiltrated to subendothelial space [3]. Prenatal hypoxia can program cardiovascular dysfunction via oxidative stress in rat offspring, and this could be ameliorated by maternal supplementation with vitamin C [4–7]. However, whether and how hypoxia during pregnancy induces vascular changes toward the development of atherosclerosis as well as possible underlying mechanisms are still unknown. Thus, the present study focused on influence of prenatal hypoxia on large blood vessels in fetal and offspring rats, as well as possible mechanisms that may contribute to formation of the early stage of atherosclerosis. Based on accumulated evidence and our preliminary testing, we hypothesized that hypoxia may activate HIF-1α; then HIF-1α could stimulate NFκB expression, followed by an increased expression of pro-inflammatory cytokines, leading to early development of atherosclerosis in the offspring.

It is well known that hypoxia could cause atherosclerosis, obstructive sleep apnea syndrome (OSAS), preeclampsia, hypertension, and other diseases [3,8,9]. In general, hypoxia activates hypoxia-inducible factor 1(HIF-1), HIF-1 is comprised of HIF-1α and

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HIF-1β [10,11]. HIF-1α is expressed dependent on oxygen levels and rapidly degraded under normoxic condition (21% O_2). However, it is stable under hypoxic condition and translocates into the nucleus mediated by nuclear localization signals. On the other hand, HIF-1β is constitutively expressed under hypoxic conditions, and heterodimerize with HIF-1α to induce transcription of over 70 genes, among which is NFκB [10–13]. Besides HIF-1α as a primarily transcription factor, NFκB also is an important regulator in immunity, inflammation, and angiogenesis [14–16]. Generally, NFκB is composed of five subunits: NFκB1 (p50 and its precursor p105), NFκB2 (p52 and its precursor p100), c-Rel, RelA (p65), and RelB; these subunits usually form the homo or hetero-dimers to execute functions in neo-vascular formation, inflammation, immunity, and apoptosis [17]. Over all, the main form of NFκB to execute functions is heterodimer of NFκB1 (p50) and RelA (p65). The heterodimer was demonstrated to induce transcription of pro-inflammatory cytokines in the pathogenesis of atherosclerosis [18].

Inflammation is among many causes, including endothelial injury; lipid infiltration; and proliferation of media smooth muscle cells that can be attributed to atherosclerosis [19]. Notably, IL-1β and TNF-α are regarded as the most important pro-inflammatory cytokines [20]. The present study also used human umbilical vein endothelia cells (HUVEC), as well as the offspring aortas, to detect the expression of HIF-1α, NFκBp65, IL-1β, and TNF-α under the hypoxic condition.

2. Material and methods

2.1. Animal preparation

All experimental procedures were approved by the Ethical Committee of Soochow University. Sprague-Dawley rats were purchased from the Animal Center of Soochow University. Rats were mated at 4 month of age. The presence of vaginal smear was designated as the first gestational day (GD). On GD 5, pregnant rats were randomly divided into two groups: the control (CON, 21% oxygen) and hypoxia group (HY, 10.5% oxygen). Hypoxia was induced by a mixture of nitrogen gas and air in an individual chamber at room temperature. All animals were fed with standard rat chow *ad libitum*. Fetal studies were carried out on GD 21 ($n = 8$ maternal rats, each group). The pregnant rats were anaesthetized using 4% chloral hydrate (0.8 mL/Kg) intraperitoneally. Male rat offspring were used at 5-month-old.

2.2. Histochemical observations

At 5 month of age, the offspring were anaesthetized, and the thoracic aorta was collected immediately, and fixed in 2.5% glutaraldehyde solution buffer at pH 7.2 with 0.1 mol/L phosphate-buffered saline, then kept at 4 °C for transmission electron microscopic studies.

2.3. Serum examination

Offspring rats were anaesthetized; blood samples were collected from the abdominal aorta with heparin sodium. For fetuses, blood samples were collected after decapitation. Serum was used for determination of triglyceride (TG), total cholesterol (TC), low-density lipoprotein-cholesterol (LDL-C), oxidized low-density lipoprotein-cholesterol (ox-LDL-C), and high-density lipoprotein-cholesterol (HDL-C). TG, TC, LDL-C, and HDL-C were determined by an automatic spectrophotometer according to the manufacturer's protocol, and all testing kits were purchased from Nanjing Jiancheng Bioengineering Institute. In addition, ox-LDL-C was determined by an Elisa kit using an automatic spectrophotometer following a standard protocol.

2.4. Plasmid construction

pCDNA3.1 plasmid was donated by Dr. Lei Zhe (Soochow university), human HIF-1α fragment (GenBank No: 000014) was amplified from pGEM-T- HIF-1α plasmid (purchased from Sino Biological Lnc, China), and digested with *Kpn*I and *Eco*RV enzyme, then cloned into the same digestion of pCDNA3.1 plasmid to construct plasmid pCDNA3.1-HIF-1α, which was identified by enzyme digestion of *Kpn*I and *Eco*RV.

2.5. Cell culture and transient transfection

HUVEC cells derived from the Human umbilical vein endothelial were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM), as endothelial injury is the initiating stage of atherosclerosis, so in the present study, HUVEC cell line is used. High Glucose supplemented with 10% (v/v) heat-inactivated fetal bovine serum (HyClone, Utah State, USA). The DMEM medium containing 100 μg/ml penicillin G and 100 μg/ml streptomycin sulfate. For transient transfection, HUVEC cells were plated in 10 cm dishes a night before transfection. Thereafter, under the condition of 1% O_2, HUVEC cells were transfected with pCDNA3.1-HIF-1α (1 μg) or pCDNA3.1 (1 μg) plasmid in the presence of jetPEI™-HUVEC (Polyplus, Strasbourg, France), which was specially designed for HUVEC transfection. At 48 h post-transfection, HUVEC cells were subjected to RNA extraction, and used for determining the mRNA expression level of NFκBp65 and pro-inflammatory cytokines by Real-time Quantitative PCR.

2.6. Luciferase reporter assay

The NFκBp65 promoter was cut into four fragments containing 1–4 hypoxia response element (HRE), following with a *Luc* gene to generate the firefly luciferase reporter plasmid pNFκBp65-1-Luc, pNFκBp65-2-Luc, pNFκBp65-3-Luc, and pNFκBp65-4-Luc, respectively. HUVEC cells were co-transfected with the plasmid pNFκBp65-Luc (0.5 μg) and the pCDNA3.1-HIF-1α (0.5 μg) or pCDNA3.1 (0.5 μg) plasmid using jetPEI™-HUVEC at 21% O_2 condition. At 48 h post-transfection, HUVEC cells were harvested and the luciferase activities were measured by a luminometer (Promega, USA) using a Dual-Lucy Assay Kit (promega, USA). All the experiments mentioned above were carried out with co-transfection with an internal control plasmid *Renilla* pRL-TK (0.1 μg). Each experiment was carried out in triplicate, and the data represent means ± SD after normalization to *Renilla* activity.

2.7. Real-time Quantitative PCR (Q-PCR)

Total RNA of offspring aorta and HUVEC cells was treated with DNaseI to remove possible contamination using RNA Isolation Kit (TaKaRa, Dalian, China). cDNA was synthesized by Prime-Script™ Reverse Transcriptase (TaKaRa, Dalian, China) with standard protocols. Q-PCR was performed to detect expression levels of NFκBp65 and pro-inflammatory cytokines. A 20-μl volume containing 0.2 μg cDNA, 5 pmol of each primer, and 10 μL of SYBR Green Real-time PCR Master Mix (TaKaRa, Dalian, China) was used for Q-PCR reaction. Q-PCR was carried out using a real-time reverse transcription (RT)-PCR System (Bio-Rad iQ™-5) according to the following program: one cycle at 95 °C for 10 min; 40 cycles at 95 °C for 15 s, 60 °C for 30 s; 81 cycles for dissociation at 55 °C for 30 s. This was repeated three times.