Curcumin inhibits hypoxia inducible factor-1α-induced inflammation and apoptosis in macrophages through an ERK dependent pathway

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Abstract. – OBJECTIVE: Atherosclerosis, a kind of peripheral arterial disease with chronic inflammation, leads to the dysfunction of the vascular system and many other diseases. Hypoxia has been proven to participate in the progression of atherosclerosis, while curcumin can inhibit hypoxia-inducible factor 1α (HIF-1α). However, the underlying mechanisms are still elusive.

PATIENTS AND METHODS: qRT-PCR was used to examine the expression of HIF-1α, IL-6 and TNFα of macrophages under hypoxic condition. Western blot was applied to examine the changes of HIF-1α, ERK and p-ERK after treatment with curcumin. Oli Red O staining and enzymatic assay were used to examine the lipid and total cholesterol in macrophages, respectively. ELISA was used to examine the release of IL-6 and TNFα by macrophages. FACS and MTT assays were applied to examine the apoptosis and proliferation of macrophages.

RESULTS: Here, we found curcumin inhibited the expression of HIF-1α at the protein level in macrophages under hypoxic condition and curcumin and HIF-1α inhibitors repressed the total cholesterol and lipid level in macrophage under hypoxic condition. Moreover, curcumin also decreased the expression of HIF-1α downstream genes, VEGF, HMOX1, ROS and PDGF. Then, the data show the HIF-1α-induced apoptosis and inflammation of macrophages were inhibited by curcumin. Curcumin also rescued the proliferation defect of macrophages caused by hypoxia. Furthermore, we found it inhibited the expression of HIF-1α via ERK signaling pathway.

CONCLUSIONS: We describe that curcumin inhibited the HIF-1α-induced apoptosis and inflammation of macrophages via ERK signaling pathways. These results suggest curcumin can be used for the treatment of atherosclerosis.

Key Words: Atherosclerosis, HIF-1α, Curcumin, Macrophage.

Abbreviation
ASO, atherosclerosis obliterans; ELISA, enzyme-linked immunosorbent assay; ERK, Extracellular Signal-regulated Kinase; FACS, fluorescence-activated cell sorting; HIF-1α, hypoxia-inducible factor 1α; HMOX1, heme oxygenase 1; IL-6, Interleukin 6; L-NAME, N-acetyl-L-leucyl-L-leucyl-L-norleucinal; qRT-PCR, quantitative real-time Polymerase Chain Reaction; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PDGF, Platelet-derived growth factor; ROS, Reactive oxygen species; TBARS, thiobarbituric acid-reactive substances; TNFα, Tumor necrosis factor α; VEGF, vascular endothelial growth factor.

Introduction
Atherosclerosis, which may cause the devastating myocardial infarction and stroke, is a progressive disease characterized by the accumulation of lipid and fibrous elements in the large arteries1-3. Atherosclerosis mostly occurs near the vascular bends and bifurcations where the turbulent blood flow generates low shear stress4, leading to the activation of endothelial cells which increases the permeability of blood vessels5. All of these changes will increase the sub-endothelial retention of lipoproteins, especially apoB-containing lipoprotein, which initiates the atherosclerosis via eliciting the immune response in the intima6-8. To response to the apoB-containing lipoproteins, macrophages and other innate immune cells enter the intima to form foam cells via engorging the lipid, causing the accumulation of lipid-rich necrotic debris in smooth muscle cells9. Next, the atheromatous plaques are formed by smooth muscle cells and lipid-rich necrotic core enclosed by the extracellular matrix1. As the disease progresses, the plaques will become
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large enough to block flow, leading to atherosclerosis obliterans (ASO), which may cause death. Although the pathology has been studied for many years and many different mechanisms have been demonstrated, the exact molecular mechanisms leading to the incurable atherosclerosis are still unknown. Hypoxia-inducible factor 1α (HIF-1α) is a transcriptional factor which can be induced by hypoxia that has been proved to participate in the progression of atherosclerosis. HIF-1α can regulate a lot of physiological and pathological functions, including angiogenesis, inflammatory response, nitric oxide metabolism, glucose metabolism and so on. It’s known that atherosclerotic lesions contain hypoxic areas, leading to the overexpression of HIF-1α in human and mouse plaques. HIF-1α has been proven to participate in the progression of atherosclerosis. For example, HIF-1α induces netrin-1/Unce expression in atherosclerotic plaques to increase the retention and survival of macrophages in intima to promote the progression of atherosclerosis. HIF-1α in macrophages accelerates atherosclerotic development via affecting the intrinsic inflammation. Since HIF-1α is overexpressed in atherosclerosis patients, HIF-1α is a good target for the treatment of atherosclerosis and the inhibitors for HIF-1α should be good candidates. Curcumin is the active ingredient of turmeric. Curcumin is commonly used in Indian traditional medicine in the treatment of biliary disorders, cough, diabetic ulcers, hepatic disorders, rheumatism and sinusitis. The paste of curcumin mixed with lime has been a popular home remedy for the treatment of inflammation and wounds. Studies demonstrate curcumin can inhibit the progression of atherosclerosis. Curcumin reduces the lipid lesion area in the entire aorta and effectively retards the occurrence and development of atherosclerotic plaques. Long-term curcumin administration protects against atherosclerosis through hepatic regulation of lipoprotein cholesterol metabolism. Curcumin reduces atherosclerosis and fatty liver by suppressing aP2 and CD36 expression in macrophages. Furthermore, curcumin has been proven to inhibit the expression of HIF-1α in pituitary adenomas. Curcumin protects liver fibrosis via inhibiting HIF-1α via ERK-dependent pathway. However, whether and how curcumin can suppress the expression of HIF-1α in macrophages is still unknown. Here, we found curcumin reduced the protein but not the mRNA level of HIF-1α in hypoxic macrophages. The downstream genes of HIF-1α, VEGF, HMOX1, ROS and PDGF, were also reduced by curcumin treatment.

The total cholesterol and lipid in macrophages were rescued by curcumin and inhibitors of HIF-1α under hypoxic condition. We also found curcumin decreased HIF-1α-induced apoptosis and the upregulated protein level of inflammatory factors, IL-6 and TNFα, in macrophages. Furthermore, we found curcumin mediated the suppression of HIF-1α via ERK signaling pathway. Our study provided a molecular mechanism for how curcumin regulate the HIF-1α in macrophages under atherosclerosis.

Materials and Methods

Cell Culture

Human THP1 cells (ATCC, Manassas, VA, USA) were seeded in 35 mm Petri dishes at a density of 0.5×10⁶ cells per ml in Roswell Park Memorial Institute-1640 (RPMI-1640) medium containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin, and the cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. The THP-1 cells were differentiated into macrophages by the addition of 100 ng/ml PMA for 72 hours. Protease inhibitor, N-acetyl-L-leucyl-L-leucyl-L-norleucinal (LLnl) (Sigma-Aldrich, St. Louis, MO, USA) (35 μM), was used to inhibit the protease.

Normoxic and Hypoxic Conditions

Cells were incubated in hypoxic condition (2% O₂) by placing them in an in vivo hypoxia work station into which a gas mixture of 5% CO₂ and balanced nitrogen was used to keep oxygen consuming system in a 3.5 L airtight chamber and verified by an anaerobic indicator strip. For normoxia experiments, cells were incubated in a humidified incubator with a constant supply of air (21% O₂) with 5% CO₂ at 37°C.

Foam Cell Formation Assay

Ox-LDL was prepared by first dissolving LDL (0.25 mg/ml) in PBS for 24 hrs at 4°C and then incubating with 5 μM CuSO4 for 24 hrs at 37°C. To stop the reaction, 0.2 μM EDTA and 50 μM BHT were added. The extent of oxidation of ox-LDL was determined by measuring thiobarbituric acid-reactive substances (TBARS) according to the manufacturer’s instructions (Cell Biolabs, San Diego, CA, USA). The cultured peritoneal macrophages were incubated with 50 μg/ml ox-LDL for 24 hours, in the presence or absence of curcumin (40 μM) or HIF-1α inhibitor (KC7F2, 40 μM).
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(St. Louis, MO, USA). Cells were then fixed with 4% w/v paraformaldehyde for 30 min and stained with 0.3% Oil-Red O for 15 min. Images were captured using microscope.

**Detection of Total Cholesterol in Macrophages**

Cultured macrophages were exposed to hypoxia with or without treatment with curcumin (40 μM) or HIF-1α inhibitors 40 μM for 24 hrs. And then macrophages were collected and lysed to extract and measure total intracellular cholesterol using the Total Cholesterol Assay Kit (Colorimetric) (Cell Biolabs, San Diego, CA, USA), according to the manufacturer’s instructions.

**Apoptosis Assay by FACS**

After 48 hrs of treatment under hypoxic condition, cells (2×10^5) were harvested and washed twice with pre-cooled PBS. The Annexin V-FITC/PI Apoptosis kit (Life Technologies, Carlsbad, CA, USA) was utilized for detecting apoptotic cells. Briefly, 5 μl aliquots of Annexin V and 1 μl aliquots of Propidium Iodide (PI) (BD Pharmingen, Franklin Lakes, NJ, USA) buffer were added into 400 μl of binding buffer. The cells were then exposed to the mixed solution for 15 min in dark at room temperature. Samples were analyzed with FACS. Next, percentage of Annexin V positive cells was recorded as a measurement of cell apoptosis.

**Quantitative Real-Time PCR Analysis (qRT-PCR)**

Total RNA from the patient plaques or culture cells was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA (1 μg) was used for reverse transcription according to the manufacturer’s instructions (Thermo Fisher, Waltham, MA, USA). Real-time PCR was performed using SYBR green Supermix (Thermo Fisher, Waltham, MA, USA). The change in mRNA expression was calculated by the comparative change-in-cycle-method (ΔΔCt) relative to GAPDH mRNA levels. The following primers were used: HIF-1α-F: GTGAACCATTCCCTCATCCGC; HIF-1α-R: GTCCTCTCCCTTCTTGAGCACAG; IL-6-F: GTTACATCCCGGAGGCATCT; IL-6-R: GTGCCTCTTGTGCTTAC; TNFα-F: GCCTCTTCTCATGTTGCA; TNFα-R: CTGATGAGAGGGAGGCCATT; HMOX1-F: GTGCCACCAAGTTCAAGCAG; HMOX1-R: CAGCTCCTGCAACTCTCCAA; VEGF-F: CTCATGGACGGGTGAGGC; VEGF-R: CTGCTCTCCCTCTGTCCGAG; ROS-F: ACCTTATCCAGCGCATTCCA; ROS-R: AGGCAGCATTGGGACATT; PDGF-F: GGAGTCGGCATACTGCT, PDGF-R: TGTCCTGGTCTGATGTTCAA; GAPDH-F: AGGTCGGTGAAACGGATTG; GAPDH-R: TGTTAGACCATGTAGTTGAGCTCA.

**Western Blot Analysis**

Protein was extracted from the plaques or cultured cells in RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.25% sodium deoxycholate, 1% NP-40). 20 μg protein were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, proteins were transferred to polyvinylidene difluoride (PVDF) membranes, blocked in TBST containing 5% milk and blotted for HIF-1α (Santa Cruz Biotechnology, Santa Cruz, CA, USA), ERK1/2 and p-ERK1/2 (Cell Signaling Technology, Danvers, MA, USA), PDGF (BD Pharmingen, Franklin Lakes, NJ, USA), VEGF, ROS, HMOX1 and GAPDH (Abcam, Cambridge, MA, USA). Horseradish peroxidase (HRP) conjugated secondary antibodies followed by chemiluminescent substrate were used for the signal detection.

**Enzyme-Linked Immunosorbent Assay (ELISA)**

The assessment of the IL-6 and TNF-α levels in the cells was performed by ELISA, using IL-6 and TNF-α ELISA Max™ Set Deluxe (BioLegend, San Diego, CA, USA), in accordance with the manufacturer’s instructions. Briefly, one day prior to running the assay, 96-well plates were coated with the capture antibody. Following 18 h incubation at 4°C, the plates were washed with PBS containing 0.05% Tween-20 (Sigma-Aldrich, St. Louis, MO, USA) and then incubated for 1 hr at room temperature with a diluent buffer to block non-specific binding. After washing, 100 ml sample (100 mg) was added to each well and then incubated for 2 hrs at room temperature. After washing of the plates, 100 ml biotinylated detection antibody was added to each well. The plates were then incubated for 1 h, prior to further washing. Next, 100 ml avidin-horseradish peroxidase (HRP) substrate solution was added and the plates were incubated in the dark for 15 min. The reaction was stopped by the addition of 100 ml 2 N sulfuric acid, and...
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the absorbance at 450 nm and 570 nm was measured.

Statistical Analysis
The difference between two groups was analyzed by two-tailed Student’s t-test. For multiple comparisons, the one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test was used to analyze the difference. p < 0.05 was considered significant.

Results

Curcumin Inhibits the Expression of HIF-1α Under Hypoxic Condition
To investigate the effect of curcumin on the expression of HIF-1α in macrophages, we treated the macrophages under hypoxic condition with or without curcumin for 6 hrs. The results showed curcumin didn’t affect the mRNA level of HIF-1α (Figure 1A). Then we examined if the protein level of HIF-1α was regulated by curcumin. We found the upregulated protein level of HIF-1α, which was induced by hypoxia, was decreased by the treatment of curcumin with a dose-dependent manner (Figure 1B). This suggested curcumin affected the protein level of HIF-1α to regulate the function of macrophages. To prove the regulation of HIF-1α at the protein level, we treated the macrophages with 35 µM proteasome inhibitor, LLnl, together with curcumin under hypoxic condition for 2 hrs or 4 hrs. The results showed LLnl decreased the inhibition of curcumin on the protein level of HIF-1α with a time-dependent manner (Figure 1C). These data demonstrated curcumin repressed the expression of HIF-1α in macrophages under hypoxic condition via proteasome-dependent manner.

Curcumin Represses the Cholesterol and Lipid Level Induced by Hypoxia via Repressing HIF-1α
Next, we examined the total cholesterol and lipid level in macrophages under hypoxic condition. The result showed that hypoxia significantly increased the total cholesterol level in macrophage. However, both treatments with curcumin and HIF-1α inhibitor can significantly decrease the total cholesterol (Figure 2A). In addition, with Oil Red O staining, we found the lipid level in macrophages was significantly increased under hypoxic condition, while administration with curcumin and HIF-1α inhibitor significantly decre-

![Figure 1](image_url)

**Figure 1.** Curcumin inhibits the expression of HIF-1α under hypoxic condition. **(A)** The mRNA level HIF-1α is not changed under hypoxic condition with or without curcumin in macrophages. **(B)** Western blot results showing the hypoxia significantly increase the protein level of HIF-1α, which is decreased after treating with curcumin. **(C)** LLnl reverses the inhibition of HIF-1α by curcumin.
treatment with 20 or 40 µM curcumin significantly decreased the apoptosis induced by hypoxia to 17.3% and 13.9% (Figure 4A). And the inhibition was dose-dependent with the high dose of curcumin can inhibit the apoptosis more dramatically than the low dose of curcumin (Figure 4A). We also examined the cell viability with MTT assay, which showed hypoxia significantly decreased macrophage viability (from 98.5% to 57.5%), which was reversed by curcumin treatment (up to 85% for 20 µM, 93% for 40 µM), which is consistent with the apoptosis results (Figure 4B). These data demonstrated curcumin can inhibit the apoptosis induced by the hypoxia.

Curcumin Decreases the Release of IL-6 and TNF-α Induced by Hypoxia

Then, we examined if curcumin can affect the expression and release of inflammatory factor. The macrophages were treated with hypoxic condition with or without curcumin. Firstly, we examined if the curcumin can affect the macrophage apoptosis induced by hypocia. We treated the macrophages with hypoxic condition with or without curcumin. We found the apoptosis percentage increased from 8.9% to 24.7% after treatment with hypoxic condition. However, the treatment with 20 or 40 µM curcumin significantly decreased the apoptosis induced by hypoxia to 17.3% and 13.9% (Figure 4A). And the inhibition was dose-dependent with the high dose of curcumin can inhibit the apoptosis more dramatically than the low dose of curcumin (Figure 4A). We also examined the cell viability with MTT assay, which showed hypoxia significantly decreased macrophage viability (from 98.5% to 57.5%), which was reversed by curcumin treatment (up to 85% for 20 µM, 93% for 40 µM), which is consistent with the apoptosis results (Figure 4B). These data demonstrated curcumin can inhibit the apoptosis induced by the hypoxia.
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got from the qRT-PCR (Figure 5 C and 5 D). These data demonstrated curcumin can significantly inhibit the inflammation response induced by hypoxia in macrophages.

Curcumin Inhibits HIF-1α via ERK Signaling Pathway

Next, we wanted to know the molecular mechanism accounting for the regulation of HIF-1α by curcumin. Previous report found curcumin inhibited the expression of HIF-1α via ERK signaling in liver fibrosis. So we examined if this signaling pathway also participate the regulation of HIF-1α by curcumin in macrophages. By Western blot, we found hypoxic condition significantly increased the protein level of HIF-1α and p-ERK, while different doses of curcumin repressed the protein level of HIF-1α and p-ERK. High dose of curcumin treatment repressed the expression of HIF-1α more dramatically than the low dose of curcumin treatment (Figure 6 A and 6 B). These data demonstrated curcumin inhibited the expression of HIF-1α via ERK signaling pathway.

Figure 3. Curcumin inhibits the expression of HIF-1α downstream in macrophages. (A) Hypoxia significantly increases the protein level of HIF-1α downstream gene, VEGP, HMOX1, ROS and PDGF. Curcumin inhibits the expression of these proteins under hypoxic condition. (B) The statistic result of Western blot (A). The experiments were repeated for 3 times. Data are presented as mean ± SD. *p < 0.05.

Discussion

Atherosclerosis is a common progressive cardiovascular disease usually occurring in the arteries. Atherosclerosis is characterized by lipid deposition and fibrosis, which contribute to the formation of atherosclerotic plaques, which may block the blood flow to lead to stroke or even death. So it’s very important to understand the molecular and cellular mechanisms for the onset and progression of atherosclerosis. It has been proven that macrophages play important roles in the pathogenesis of atherosclerosis. Macrophage activation in atheroma leads to the release of vasoactive molecules such as nitric oxide, endothelins, and several eicosanoids. And the activated macrophages also produce reactive oxygen species for lipoprotein oxidation and cytotoxicity. Furthermore, activated macrophages secrete proteolytic enzymes which degrade matrix components, which may lead to destabilization of plaques and an increased risk for plaque rupture and thrombosis. So we wanted to explore the function of macrophages in atherosclerosis.
Previous studies indicated that hypoxic regions in atherosclerotic plaques showed higher expression of HIF-1α, which increased the formation of macrophage foam cells via regulating macrophage lipid metabolism by inducing the sterol synthesis and suppressing the cholesterol efflux. And HIF-1α in macrophages affected the intrinsic inflammatory profile of macrophages and promoted development of atherosclerosis. Furthermore, recently report showed that Hypoxia inducible factor worked as a therapeutic target for atherosclerosis. Thus, the drug that can target to HIF-1α may be a method for the treatment of atherosclerosis. Our study found curcumin repressed HIF-1α to reduce the proliferation and inflammation of macrophages. Furthermore, the treatment of curcumin significantly decreased the total cholesterol and lipid level in macrophages under hypoxic condition, which suggested curcumin can repress the effect of hypoxia on macrophages via HIF-1α signaling pathway. This provides a new way for targeting to HIF-1α. Curcumin, the main active compound in turmeric, has been studied for its use as anti-cancer, anti-aging and wound healing agent. Furthermore, studies have shown

![Figure 4](image)

**Figure 4.** Curcumin inhibits the HIF-1α-induced macrophage apoptosis. *(A)* FACS results show the apoptosis was induced under hypoxic condition, which was inhibited by treating with curcumin. *(B)* Hypoxia decreases the proliferation of macrophages, which is reversed by treating with curcumin. Data are presented as mean ± SD. *p < 0.05.
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Curcumin can be benefit for the treatment of atherosclerosis. However, the underlying mechanisms are still unknown. In our work, we found curcumin repressed the expression of HIF-1α at the protein level and the downstream genes of HIF-1α, such as HMOX1, ROS, VEGF and PDGF. The downstream genes of HIF-1α are critical for the progression of atherosclerosis. For example, VEGF can enhance atherosclerotic plaque formation and progression, PDGF can regulate the proliferation and migration of vascular smooth muscle cells which is critical for the formation of atherosclerotic lesions. ROS plays a significant role in homoeostasis of vascular cells and the pathogenesis of atherosclerosis. Thus, curcumin may be a good drug for the treatment of atherosclerosis via repressing HIF-1α. Furthermore, the activation of macrophages is important for the progression of atherosclerosis. Activated macrophages release many inflammatory factors, like IL-1, IL-6, TNFα, NO and ROS, to promote atherosclerosis. To elucidate if curcumin can repress the activation of macrophage, we examined the apoptosis, proli-
We found curcumin repressed the expression of HIF-1α at the protein level in macrophages at the molecular level. Curcumin, at the cellular level, inhibited the HIF-1α-induced apoptosis and the upregulated protein level of inflammation factors of macrophages. At last, we found curcumin repressed the expression of HIF-1α via ERK signaling pathway. This finding elucidated the mechanisms of the benefits of curcumin on atherosclerosis. Furthermore, these data indicated curcumin can be a drug for treatment of atherosclerosis. However, it will be very important and interesting to elucidate the function of curcumin for atherosclerosis in mice in vivo.

Conclusions

References


Conflict of Interest

The Authors declare that they have no conflict of interest.

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Statement

No animal and clinical samples were used in current study. The Ethics Committee approval is not provided.

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