Mechanism of Liraglutide Against Oxidized Low-Density Lipoprotein-Induced Endothelial Cell Injury Through the NLRP3 Inflammasome Pathway (Postprint)

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Abstract

Background: Atherosclerosis is the leading cause of cardiovascular and cerebrovascular diseases worldwide. Inflammation represents a major research focus, with the NOD-like receptor 3 (NLRP3) being the most extensively studied inflammasome. GLP-1 receptor agonists exhibit anti-atherosclerotic effects, though the underlying mechanisms remain unclear.

Objective: To investigate the mechanism by which liraglutide antagonizes oxidized low-density lipoprotein (ox-LDL)-induced endothelial cell injury.

Methods: Human umbilical vein endothelial cells (HUVECs) were cultured. The control group comprised HUVECs treated with blank serum; the model group comprised HUVECs treated with 100 g/mL ox-LDL for 48 h; and the low-dose, medium-dose, and high-dose liraglutide groups comprised HUVECs treated with 100 g/mL ox-LDL for 24 h followed by treatment with 100 nmol/L, 200 nmol/L, or 400 nmol/L liraglutide for 24 h, respectively. Cell proliferation rate was assessed using CCK-8 assay. Pyroptotic cell morphology was examined via scanning electron microscopy. Lactate dehydrogenase (LDH) activity was measured. Interleukin (IL)-1β and IL-18 expression levels were determined by enzyme-linked immunosorbent assay (ELISA). Protein expression levels of NOD-like receptor 3 (NLRP3), adaptor protein apoptosis-associated speck-like protein (ASC), cysteine-aspartic protease 1 (Caspase-1), pyroptosis execution protein (GSDMD), and N-terminal domain pyroptosis execution protein (N-GSDMD) were detected by Western blot.

Results: Cell proliferation rates in the model, low-dose liraglutide, and medium-dose liraglutide groups were lower than in the control group, whereas those in the low-dose, medium-dose, and high-dose liraglutide groups were higher than

in the model group (P<0.05). Scanning electron microscopy revealed significant pyroptosis in the model group, which was markedly ameliorated in the low-dose, medium-dose, and high-dose liraglutide groups. LDH activity in the model and low-dose liraglutide groups was higher than in the control group, while LDH activity in the low-dose, medium-dose, and high-dose liraglutide groups was lower than in the model group (P<0.05). IL-1β expression levels in the model and low-dose liraglutide groups were higher than in the control group, whereas IL-1β expression levels in the medium-dose and high-dose liraglutide groups were lower than in the model group (P<0.05). IL-18 expression level in the model group was higher than in the control group, while IL-18 expression levels in the low-dose, medium-dose, and high-dose liraglutide groups were lower than in the model group (P<0.05). Expression levels of NLRP3, ASC, Caspase-1, GSDMD, and N-GSDMD in the model group were higher than in the normal group; ASC and Caspase-1 expression levels in the low-dose liraglutide group were higher than in the normal group; NLRP3 and ASC expression levels in the medium-dose liraglutide group were lower than in the model group; and NLRP3, ASC, and Caspase-1 expression levels in the high-dose liraglutide group were lower than in the model group (P<0.05).

Conclusion: Liraglutide significantly inhibits ox-LDL-induced NLRP3 inflammasome activation in endothelial cells and suppresses endothelial cell pyroptosis, thereby exerting anti-atherosclerotic effects.

Full Text

Mechanism of Liraglutide in Oxidized Low-Density Lipoprotein-Induced Endothelial Cell Injury Based on the NOD-Like Receptor 3 Inflammasome Pathway

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Abstract

Background: Atherosclerosis is the leading cause of cardiovascular and cerebrovascular diseases worldwide, with inflammation representing a major research focus. Among inflammatory pathways, the NOD-like receptor 3 (NLRP3) inflammasome is the most extensively studied. GLP-1 receptor agonists have demonstrated anti-atherosclerotic effects, though the specific mechanisms remain unclear.

Objective: To investigate the mechanism by which liraglutide antagonizes oxidized low-density lipoprotein (ox-LDL)-induced endothelial cell injury.

Methods: Human umbilical vein endothelial cells (HUVECs) were cultured and divided into five groups: a control group treated with blank serum, a model group treated with 100 g/mL ox-LDL for 48 hours, and three liraglutide treatment groups that received 100 nmol/L, 200 nmol/L, or 400 nmol/L liraglutide for 24 hours following 24-hour ox-LDL exposure. Cell proliferation rates were calculated using the CCK-8 assay. Pyroptotic cell morphology was examined via scanning electron microscopy. Lactate dehydrogenase (LDH) activity was measured, and interleukin (IL)-1β and IL-18 expression levels were detected by enzyme-linked immunosorbent assay (ELISA). Western blotting was used to assess protein expression levels of NLRP3, apoptosis-associated speck-like protein containing a CARD (ASC), caspase-1, gasdermin D (GSDMD), and N-terminal GSDMD (N-GSDMD).

Results: Cell proliferation rates in the model group and low- and medium-concentration liraglutide groups were lower than in the control group, while all liraglutide-treated groups showed higher proliferation rates compared to the model group ($P<0.05$). Scanning electron microscopy revealed prominent pyroptosis in the model group, with significant improvement observed across all liraglutide-treated groups. LDH activity was elevated in the model group and low-concentration liraglutide group compared to controls, but reduced in all liraglutide groups versus the model group ($P<0.05$). IL-1β expression was higher in the model group and low-concentration liraglutide group than in controls, while medium- and high-concentration liraglutide groups showed lower IL-1β levels than the model group ($P<0.05$). IL-18 expression was elevated in the model group compared to controls, but reduced across all liraglutide-treated groups ($P<0.05$). The model group exhibited higher expression of NLRP3, ASC, caspase-1, GSDMD, and N-GSDMD than the normal group. The low-concentration liraglutide group showed higher ASC and caspase-1 expression than controls, while the medium-concentration group had lower NLRP3 and ASC expression than the model group, and the high-concentration group demonstrated reduced NLRP3, ASC, and caspase-1 expression compared to the model group ($P<0.05$).

Conclusion: Liraglutide significantly inhibits NLRP3 inflammasome activation and pyroptosis in endothelial cells induced by ox-LDL, exerting anti-atherosclerotic effects.

Keywords: atherosclerosis; liraglutide; endothelial cells; oxidized low-density lipoprotein; NOD-like receptor 3

1. Materials and Methods

1.1 Experimental Period

March 25, 2022 to May 19, 2022.

1.2 Cells and Main Reagents

Liraglutide injection was purchased from Novo Nordisk (catalog number: 8074252). Human umbilical vein endothelial cells (HUVECs) were obtained from Procell (catalog number: CL-0122) along with HUVEC-specific culture medium. The lactate dehydrogenase (LDH) activity assay kit was from Solarbio (catalog number: BC0685). Antibodies included β-actin (internal reference, rabbit anti) (Bioss, catalog number: bs-0061R), anti-NLRP3 antibody (Bioss, catalog number: bs-10021R), anti-ASC antibody (Bioss, catalog number: bs-34024R), anti-caspase-1 antibody (Bioss, catalog number: bs-10743R), anti-gasdermin D (GSDMD) antibody (Abcam, catalog number: ab209845), and anti-N-terminal GSDMD (N-GSDMD) antibody (Abcam, catalog number: ab215203). Additional reagents comprised the BCA protein concentration assay kit (Solarbio PC0020) and high-efficiency RIPA tissue/cell lysis buffer (Solarbio R0010).

1.3 Main Instruments and Equipment

Equipment included a CO_2 incubator with air jacket and touchscreen control (CI-191X), low-speed benchtop centrifuge (L600-A), vortex mixer (GL-88D), biosafety cabinet (BSC-1304 II A2), inverted biological microscope system (ICX41), digital constant temperature water bath (HH-1), microplate reader (K6600-B), vertical high-pressure steam sterilizer (LDZX-50KBS), flow cytometer (LSRII), electron microscope (Hitachi Regulus 8100), mini vertical electrophoresis and transfer system (1645050), and low-temperature refrigerated centrifuge (H2050R).

1.4 Experimental Methods

1.4.1 Cell Culture and Treatment HUVECs were cultured in DMEM medium containing 10% fetal bovine serum at 37°C in a 5% CO_2 incubator. Upon reaching approximately 90% confluence, cells were passaged. The culture medium was discarded, cells were washed twice with PBS, and EDTA-trypsin was added for cell detachment. Detached cells were transferred to 15 mL centrifuge tubes and centrifuged at 1000 r/min for 5 minutes at room temperature. After removing the supernatant, cells were resuspended in fresh complete medium and returned to the incubator. Passages 3-7 were used for subsequent experiments.

1.4.2 Experimental Groups and Drug Administration HUVECs were divided into five groups: control, model, low-concentration liraglutide, medium-

concentration liraglutide, and high-concentration liraglutide. The control group received blank serum. The model group was treated with 100 g/mL ox-LDL for 48 hours. The three liraglutide groups received 100 nmol/L, 200 nmol/L, or 400 nmol/L liraglutide for 24 hours following 24-hour ox-LDL exposure. The ox-LDL concentration was selected based on the study by Zheng et al. [5], while liraglutide concentrations were chosen according to the method described by Gao [6].

1.5 Experimental Procedures

1.5.1 Cell Proliferation Assay (CCK-8) Cells were cultured in 96-well plates for 24 hours, then 10 L of CCK-8 solution was added to each well. After incubating for 4 hours, absorbance at 450 nm was measured using a microplate reader. Cell proliferation rate (%) = [(experimental group OD - blank group OD) / (control group OD - blank group OD)] × 100%.

1.5.2 Scanning Electron Microscopy Cell morphology was examined using scanning electron microscopy to observe pyroptotic changes.

1.5.3 Lactate Dehydrogenase (LDH) Activity Assay Cell culture supernatants were collected and centrifuged at 12,000 g for 20 minutes at 4°C. LDH activity was measured following the kit instructions, with absorbance at 450 nm determined using a microplate reader.

1.5.4 Enzyme-Linked Immunosorbent Assay (ELISA) for IL-1β and IL-18 Cell culture supernatants were centrifuged at 1,000 g for 20 minutes. IL-1β and IL-18 levels were detected according to kit protocols.

1.5.5 Western Blot Analysis of NLRP3 Inflammasome and Pyroptosis-Related Proteins Total cellular protein was extracted and quantified using the BCA protein assay kit. Samples were subjected to SDS-PAGE, transferred to membranes, blocked at room temperature, and incubated with specific primary antibodies. β-actin served as the internal reference. After chemiluminescent detection, band densities were analyzed using ImageJ 1.0 to obtain gray values and calculate target protein expression relative to the internal control. Each group was analyzed in triplicate.

1.6 Statistical Analysis

Data were analyzed using GraphPad Prism 7.0 software. Normally distributed continuous variables are presented as mean ± standard deviation (x-±s). Comparisons among multiple groups were performed using one-way ANOVA, with pairwise comparisons conducted via LSD-t test. Statistical significance was defined as $P<0.05$.

2. Results

2.1 Comparison of HUVEC Proliferation Rates Among Five Groups

Significant differences in cell proliferation rates were observed among the five groups ($P<0.05$). The model group and both low- and medium-concentration liraglutide groups showed lower proliferation rates than the control group, while all liraglutide-treated groups demonstrated higher proliferation rates compared to the model group ($P<0.05$) .

2.2 Scanning Electron Microscopy Observations

Electron microscopy revealed distinct morphological differences among groups. Control group cells exhibited intact morphology without obvious damage. In contrast, model group cells displayed prominent pyroptotic features, including membrane blebbing, swelling, rupture, fenestration, and release of intracellular contents. Liraglutide treatment attenuated pyroptosis in a dose-dependent manner: high-dose liraglutide showed the mildest pyroptosis with only localized membrane swelling and visible pseudopodia; medium-dose liraglutide showed relatively mild pyroptosis with fewer and smaller membrane ruptures and fenestrations; while low-dose liraglutide exhibited more severe pyroptosis with extensive membrane swelling and damage [Figure 1: see original paper].

2.3 Comparison of LDH Activity Among Five Groups

LDH activity differed significantly among groups ($P<0.05$). The model group and low-concentration liraglutide group showed higher LDH activity than controls, while all liraglutide-treated groups exhibited lower LDH activity compared to the model group ($P<0.05$) .

2.4 Comparison of IL-1β and IL-18 Expression Levels Among Five Groups

Significant differences were observed in IL-1β and IL-18 expression levels ($P<0.05$). IL-1β expression was higher in the model group and low-concentration liraglutide group versus controls, while medium- and high-concentration liraglutide groups showed lower IL-1β levels than the model group ($P<0.05$). IL-18 expression was elevated in the model group compared to controls, but reduced across all liraglutide-treated groups ($P<0.05$) .

2.5 Comparison of NLRP3 Inflammasome and Pyroptosis-Related Protein Expression

Significant differences were found in NLRP3, ASC, caspase-1, GSDMD, and N-GSDMD expression levels among groups ($P<0.05$). The model group exhibited higher expression of all these proteins compared to the normal group. The low-concentration liraglutide group showed higher ASC and caspase-1 expression than controls. The medium-concentration group demonstrated lower NLRP3

and ASC expression than the model group, while the high-concentration group showed reduced NLRP3, ASC, and caspase-1 expression compared to the model group ($P<0.05$) [Figure 2: see original paper].

3. Discussion

Innate immune pattern recognition receptors play a crucial role not only in atherosclerosis development but also serve as a connecting mechanism linking various immune responses throughout the atherosclerotic process [7-8]. These receptors primarily comprise two classes: cytoplasmic NOD-like receptors (NLRs) and cell membrane Toll-like receptors (TLRs). The NLRP3 inflammasome is an intracellular pattern recognition receptor and the most extensively studied inflammatory complex, consisting of receptor protein NLRP3, adaptor protein ASC, and effector protein pro-caspase-1 [9].

Upon stimulation by various signals, activated NLRP3 expression increases significantly, recruiting ASC and interacting to convert inactive pro-caspase-1 into active caspase-1, which then exerts its effector functions [10]. Activated caspase-1 cleaves substrates such as pro-IL-1, promoting the maturation and secretion of inflammatory mediators IL-1β and IL-18 [11]. Throughout atherosclerosis pathogenesis, IL-1β and IL-18 serve as crucial mediators in innate immune responses and master regulators of inflammatory reactions, not only participating directly in inflammation but also acting as upstream factors triggering downstream cascades of other inflammatory cytokines [12].

The NLRP3 inflammasome promotes caspase-1 activation, leading to a form of programmed cell death known as pyroptosis [13], characterized by cell membrane rupture and release of intracellular inflammatory contents [14]. In the canonical pyroptosis pathway, different NLRs selectively respond to corresponding stimuli, after which pro-caspase-1 connects with NLR receptor proteins via ASC to form high-molecular-weight complexes. This complex undergoes autocleavage to generate enzymatically active caspase-1, which cleaves GSDMD to release N-GSDMD. The N-GSDMD fragment then binds to membrane lipids, oligomerizes, and forms pores in the cell membrane, causing cell swelling and pyroptotic death [15-17]. These membrane pores facilitate the release of pro-inflammatory factors such as IL-1β and IL-18, amplifying the inflammatory response [18-19]. Studies have detected abundant caspase-1 in ruptured atherosclerotic plaques, further confirming that NLRP3 inflammasome-mediated pyroptosis participates in atherosclerosis progression [20].

In our study, the model group showed reduced cell proliferation and increased LDH activity, confirming successful model establishment. Liraglutide antagonized ox-LDL-induced endothelial cell injury, as evidenced by improved cell proliferation rates and reduced membrane damage. Compared with the model group, IL-1β and IL-18 levels were significantly decreased in the medium- and

high-concentration liraglutide groups, indicating that liraglutide at appropriate concentrations can suppress inflammatory responses.

Furthermore, expression of inflammasome-related proteins (NLRP3, ASC, caspase-1) and pyroptosis-related proteins (GSDMD, N-GSDMD) was elevated in the model group compared to controls, confirming that inflammatory responses and pyroptosis participate in atherosclerosis and validating our model. NLRP3, ASC, and caspase-1 expression was reduced in the high-concentration liraglutide group compared to the model group, while ASC expression was decreased in the medium-concentration group, demonstrating that high-dose liraglutide can inhibit inflammasome expression.

As research on atherosclerosis deepens, investigators continue to explore its pathogenic mechanisms. Excessive lipid deposition leads to plaque formation, while inflammatory responses trigger pro-inflammatory cytokine release and mediate pyroptosis, thereby accelerating atherosclerosis progression [21]. Our study demonstrates that liraglutide significantly inhibits NLRP3 inflammasome activation and reduces endothelial cell pyroptosis induced by ox-LDL, thereby exerting anti-atherosclerotic effects. These findings may provide novel insights and therapeutic targets for clinical atherosclerosis treatment. Due to funding limitations, this study did not include animal experiments or investigate the specific activation mechanisms of the NLRP3 inflammasome, which represent future research directions.

Author Contributions

CHEN Ling conceptualized the study, designed the research, conducted the experiments, and wrote the manuscript. XU Rui performed experimental operations and collected and organized data. ZHANG Zhanying conducted statistical analysis and prepared figures and tables. CHEN Xinchun revised the manuscript. XU Hong was responsible for quality control, overall manuscript supervision, and project management.

Conflicts of Interest: None declared.

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