

Original Article

Effects and mechanisms of indol-2, 3-dione on atherosclerosis

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Received May 24, 2014, Accepted June 30, 2014; Epub August 15, 2014; Published August 30, 2014

Abstract: Purpose: Indol-2, 3-dione (ISA), a natural substance with clear structure, has been shown to process anti-inflammatory, antioxidant and anti-atherosclerosis activity in vivo. The purpose of this study was to investigate the effect and mechanism of ISA on AS by primary rat cardiac microvascular endothelial cells (rCMEC). Methods: rCMEC cells were primary cultured, appraised by cell morphology and immune cell chemical dyeing, and passaged to the 3rd generation. The effect of ISA on the activity of rCMEC induced by oxidized low-density lipoprotein (ox-LDL) was detected by MTT method. Then we studied the effect of ISA on the adhesion of monocyte with rCMEC induced by ox-LDL. The secretion of interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) by rCMEC were measured by enzyme-linked immunosorbent assay (ELISA) method. Finally the mRNA level of IL-6 and TNF- α of rCMEC were analyzed by real time RT-PCR. Results MTT result indicated that ISA (10^{-8} - 10^{-6} g/L) could inhibit rCMEC injury induced by ox-LDL in a dose-dependent manner ($P < 0.05$). The adhesion of monocyte with rCMEC induced by ox-LDL was inhibited by ISA in a dose-dependent manner ($P < 0.05$). The levels of IL-6 and TNF- α of ISA groups were significantly decreased in a dose-dependent manner compared with model group ($P < 0.05$). The mRNA expressions of IL-6 and TNF- α of ISA groups were also downregulated significantly compared with model group ($P < 0.05$). Conclusions ISA can prevent atherosclerotic lesion. Its mechanism may be that it can defend against the oxidation damage to rCMEC, inhibit the adhesion of monocyte to rCMEC, and reduce inflammatory secretions of IL-6 and TNF- α .

Keywords: Indol-2, 3-dione, atherosclerosis, ox-LDL, IL-6, TNF- α

Introduction

Cardiovascular disease is the biggest killer globally and the principal contributing factor to the pathology is atherosclerosis; a chronic, inflammatory disorder characterized by lipid and cholesterol accumulation and the development of fibrotic plaques within the walls of large and medium arteries [1]. Either by secretion of inflammatory mediator or by modulation of expression of adhesive molecules on vascular surface, vascular endothelial cells play a key role in initiation and amplification of atherogenesis.

In 1989, Steinberg et al. [2] put forward the original oxidative modification hypothesis based on the notion that oxidation represents a biologic modification analogous to chemical modification discovered to give rise to foam cells. Since then, numerous studies have sup-

ported the ox-LDL hypothesis which says ox-LDL can promote foam cell formation through the lectin-like oxidized LDL receptor-1 (LOX-1 receptor) [3]. Recently, it was recognized that activation of LOX-1 receptor could lead to the increase IL-6 and TNF- α secretions, which play a key role for the endothelial cell dysfunction [1, 4, 5].

Indol-2, 3-dione (ISA) is widely distributed in plants, animals and humans, it is necessary marine active substances to maintain the lobster survive, it's one of the active ingredients of traditional Chinese medicine indigo naturalis and folium isatidis [6]. It was firstly isolated from urine in human, then it was found in other tissues such as seminal fluid, brain and heart [7]. Previous studies have demonstrated ISA is the inhibitor of MAO, and it has many biological effects [8], such as anti-inflammatory effect, anti-oxidation effect and anti-atherosclerosis.

Our study has demonstrated that ISA can resist the onset and progression of atherogenesis in quail. It has high efficiency and low toxicity, maybe it is a potential natural medicine.

Material and methods

rCMEC primary culture and administration

Referred to Nishida's method [9], rCMEC cells were primary cultured. The rCMECs were identified by cell morphology and immune cell chemical dyeing. Cells were cultured in 25 mm² corning flask in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine solution and 100 µg/ml penicillin/streptomycin. The 3rd generation of cells was used in the subsequent experiment.

Cell viability assay

The cells were replaced to 96-well microplates for incubation for 24 hours at 37°C, 5% CO₂. After pretreatment with ISA for 24 hours, the rCMEC was incubated with ox-LDL (0.1 g/L) for next 24 hours. After suction of the liquid in the wells, MTT was added to a final concentration of 0.5 mg/ml, and incubation was continued for 4 h at 37°C, 5% CO₂. After suction of MTT, dimethylsulfoxide (DMSO 150 µl per well) was added and mixed fully, the optical density was detected by enzyme linked immune detector at 570 nm as cell viability.

Adhesion of mononuclear cell to rCMEC

The cells were replaced to 96-well microplates for incubation for 24 hours at 37°C, 5% CO₂. After pretreatment with ISA for 24 hours, the rCMECs were incubated with ox-LDL (0.1 g/L) for another 24 hours. At the same time the mononuclear cell was prepared according to the introduction, and the cell density was adjusted as 1×10^6 ml⁻¹ by DMEM. After suction of the liquid in the wells, mononuclear cell suspension (150 µl per well) was added, following incubation at 37°C for 1 h. After washed with phosphate buffer solution (PBS), rose Bengal (0.25%, 150 µl per well) was added to affect the cells (room temperature, 5-10 min). After washed with PBS, PBS-alcohol (1:1, 200 µl per well) was added (20°C 30 min). Then the optical density was detected by enzyme linked immune detector at 570 nm. The optical density is proportional to the number of mononuclear cells.

Secretion of IL-6 and TNF-α

The cells were replaced to 6-well plates for incubation for 24 hours at 37°C, 5% CO₂. After pretreatment with ISA for 24 hours, rCMECs were incubated with ox-LDL (0.1 g/L) for another 24 hours.

IL-6 and TNF-α levels in the cultural supernatants were detected by an ELISA kit according to the instruction provided. The optical density of the samples was read on a microplate reader at 450 nm.

Total RNA of the cells was extracted with Trizol according to the manufacturer's instruction. cDNA were synthesized using PrimeScript[®]RT reagent Kit according to the manufacturer's instruction. The PCR reaction mixture contained 12.5 µl SYBR[®] Premix Ex TaqTM, 10 µM of PCR Forward Primer and PCR Reverse Primer, 2 µl cDNA and 8.5 µl dH₂O, adding up to a final volume of 25 µl. The β-actin was selected as a housekeeping gene. The amplification procedure consists of 42 cycles (94°C, 30 s; 50°C, 60 s; 68°C, 120 s) with the following oligonucleotide primer sets:

IL-6 sense: 5'-TCCGTTTCTACCTGGAGTTTGT-3', IL-6 antisense: 5'-GTTGGATGGTCTTGGTCC-TTAG-3', TNF-α sense: 5'-CATGGATCTCAAAGAC-AACCAA-3', TNF-α antisense: 5'-CTCCTGGTATG-AAATGGCAAAT-3', β-actin sense: 5'-CACCCG-CGAGTACAACCTTC-3', β-actin antisense: 5'-CCCATACCCACCATCACACC-3'.

The threshold cycle, ΔCt, was calculated as Ct (target gene) - Ct (β-actin). The relative changes in target gene in different treatment groups were determined by the formula $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct (\text{control}) - \Delta Ct (\text{treatment group})$.

Statistical analysis

The experiment data were expressed in mean ± SD and analyzed using ANOVA (IBM SPSS software, 19.0 v), LSD-t test was applied to analyze the difference between individual group. $P < 0.05$ were considered statistically significant.

Results

Effect of ISA on cell viability

As shown in **Figure 1**, exposure of the cells to ox-LDL for 24 h significantly decreased the cell viability compared with control group ($P < 0.05$). However, ISA can inhibit cell injury made by ox-

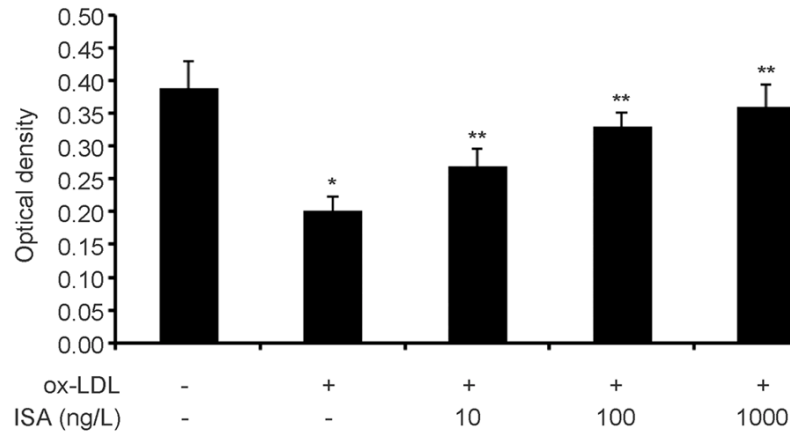


Figure 1. Effect of ISA on cell viability. The rCMEC were pretreated for 24 h with different concentrations of ISA, and then exposed to ox-LDL (0.1 g/L) for another 24 h. Cell viability was evaluated by the MTT method, which quantified the cell viability as the optical density at 570 nm. * $P < 0.05$ vs. control, ** $P < 0.05$ vs. ox-LDL.

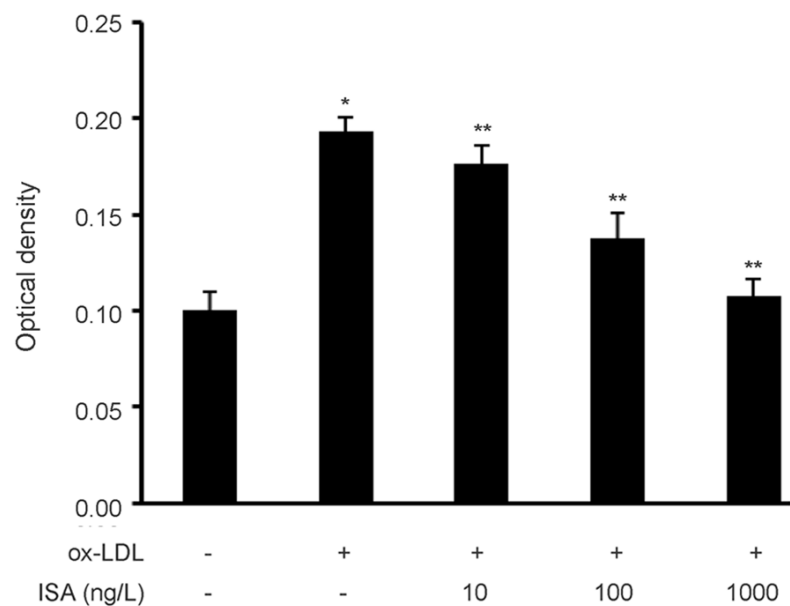


Figure 2. Effect of ISA on the adhesion of mononuclear cell to rCMEC. The rCMEC were pretreated for 24 h with different concentrations of ISA, and then exposed to ox-LDL (0.1 g/L) for another 24 h. The adhesion of mononuclear cell to rCMEC was quantified as the optical density at 570 nm. * $P < 0.05$ vs. control, ** $P < 0.05$ vs. ox-LDL.

LDL in a dose-dependent manner, and have significant difference ($P < 0.05$) compared with model group.

Effect of ISA on the adhesion of mononuclear cell to rCMEC

As shown in **Figure 2**, exposure of the cells to ox-LDL for 24 h significantly increased the ad-

hesion of mononuclear cell to rCMEC compared with control group ($P < 0.05$). However, ISA significantly inhibited cell adhesion caused by ox-LDL in a dose-dependent manner compared with model group ($P < 0.05$).

Effect of ISA on secretion of IL-6 and TNF- α

As shown in **Figure 3**, exposure of the cells to ox-LDL for 24 h significantly increased the content of IL-6 and TNF- α in culture fluid compared with control group ($P < 0.05$). However, ISA significantly inhibited the secretion of IL-6 and TNF- α caused by ox-LDL in a dose-dependent manner compared with model group ($P < 0.05$).

The RT-PCR result is as shown in **Figure 4**, ox-LDL induced a significant increase in expression of IL-6 and TNF- α compared with control group ($P < 0.05$). However, ISA significantly inhibited the expression of IL-6 and TNF- α , and have significant difference ($P < 0.05$) compared with model group.

Discussion

Atherosclerosis is characterized by complicate mechanisms. During recent years, the role of inflammation in atherosclerosis has been increasingly recognized [1, 5]. Under pathological conditions, endothelial cells (ECs) lesion is thought to play a fundamental role in the development of atherosclerosis by inducing neointima formation [10], inflammatory cell infiltration [11], lipid transport [12], plaque rupture [13], and release cytokines [14]. Moreover, when the development of atherosclerosis comes to the late stage of the formation of ath-

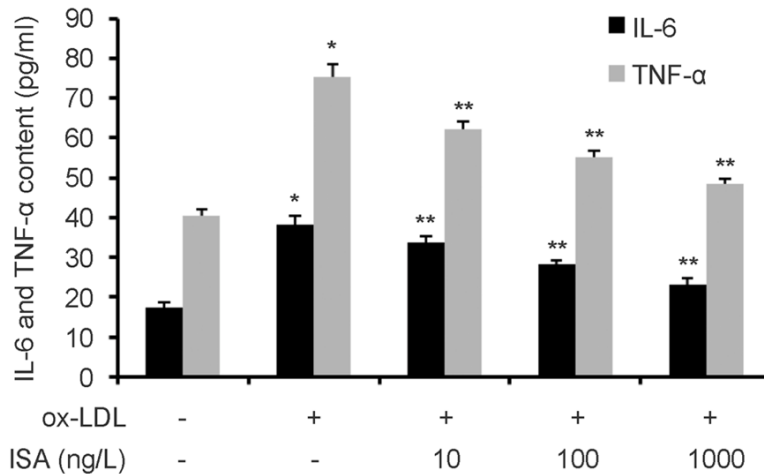


Figure 3. Effect of ISA on secretion of IL-6 and TNF- α . The rCMEC were pretreated for 24 h with different concentrations of ISA, and then exposed to ox-LDL (0.1 g/L) for another 24 h. IL-6 and TNF- α content was evaluated by the ELISA method, which quantified the content as the optical density at 450 nm. * $P < 0.05$ vs. control, ** $P < 0.05$ vs. ox-LDL.

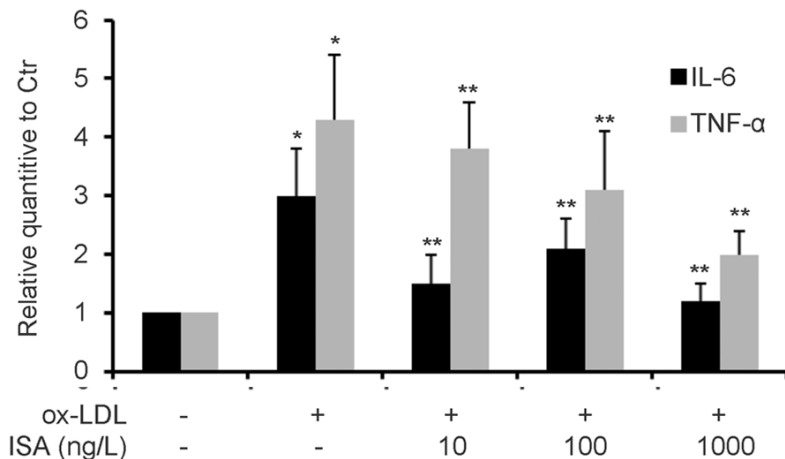


Figure 4. Effect of ISA on expression of IL-6 and TNF- α . * $P < 0.05$ vs. control, ** $P < 0.05$ vs. ox-LDL.

eromatous plaque, ECs are the most numerous of the immune cells populating the plaque [15]. That is to say the ECs lesion run through the onset and development of atherosclerosis, and is a critical in atherosclerosis. So we chose rCMEC for the experiment and established the primary culture method in vitro.

Among the risk factors for endothelium injury, ox-LDL is now considered to be a key atherosclerotic risk factor [16], and contributes greatly to the development and progression of atherosclerosis. It induces the ECs shrinkage, increases expression of adhesion molecules on

ECs, changes the secretory activities of endothelium, reduces the antioxidant capability [17], and enhances ECs apoptosis [18]. Previous studies have shown that ox-LDL induces ECs apoptosis by decreasing the expression of anti-apoptotic gene B-cell CLL/lymphoma 2 (Bcl-2) [19, 20]. When treated by ox-LDL, ECs can express cell adhesion molecules, which cause the adherence of monocytes and lymphocytes to the ECs [21]. At the same time, they can also express all kinds of cytokines, such as IL-6, TNF- α , and IL-1, which are important mediators of atherosclerosis.

In this study, the rCMEC was incubated with ox-LDL, the cell viability was decreased and the adhesion to mononuclear cell was increased significantly, ISA can inhibit cell injury and adhesion made by ox-LDL in a dose-dependent manner. It showed ISA can attenuate the impairment induced by ox-LDL, reduce the adhesion ability of rCMEC to inflammatory cells. At the same time, ISA decrease the secretion and expression of IL-6 and

TNF- α activated by ox-LDL, which are important inflammatory factors.

ISA exhibit a protective effect on endothelial cell injury caused by ox-LDL, and the exact mechanism is associated with anti-adhesion and anti-inflammation.

Acknowledgements

This work was supported by National Major Scientific and Technological Special Project for "Significant New Drugs Development" of China (No. 2009ZX09102-050) and Natural Science

Foundation of Shandong Province (No. ZR2010-HM129).

Disclosure of conflict of interest

None.

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