Original Article

TLR2 expression doesn’t change in ox-LDL mediated inflammation in Human umbilical vein endothelial cells under high glucose culture

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Abstract: Background: Inflammatory responses induced by ox-LDL play important roles in atherogenesis, and could be promoted in diabetic patients. Toll-like receptor (TLR)2 is an innate inflammatory receptor, and is enhanced in human umbilical vein endothelial cells (HUVECs) under high glucose conditions. Ox-LDL-TLR2 pathway activation and further inflammation in monocytes are involved in the atherosclerosis formation. Objective: What role of TLR2 plays on ox-LDL-induced inflammation in HUVECs remains unclear, especially in high glucose conditions. The purpose of this study is to explore the effect and role of ox-LDL-TLR2 pathway on the inflammatory responses in HUVECs. Methods: 1 hour prior to the treatment, HUVECs were treated with or without neutralizing anti-TLR2 antibody. After that, HUVECs were treated with ox-LDL (20, or 40 μg/ml) or LPS (200 ng/ml) under normal and high glucose conditions. The expressions of ICAM-1 and TLR2 protein were analyzed by immunoblotting, and IL-6 and IL-8 were measured by ELISA. Results: Compared with those in normal glucose condition, IL-6 and IL-8 expression were increased in high glucose condition. The stimulation of ox-LDL and LPS both increased the expression of ICAM-1, IL-6 and IL-8, but did not change TLR2 protein expression in both normal and high glucose conditions. Additionally, the expression of ICAM-1, IL-6 and IL-8 was not changed when TLR2 was knocked out under these two conditions. Conclusion: The inflammatory responses induced by ox-LDL were not changed with or without TLR2 under both normal and high glucose conditions in HUVECs. Our study indicates TLR2 is not involved in the ox-LDL mediated endothelial injury under high glucose conditions, which is an important step of atherosclerosis formation in diabetes.

Keywords: TLR2, inflammation, HUVECs, ox-LDL

Introduction

Diabetes mellitus is an important risk factor for the development of atherosclerosis. Endothelial dysfunction induced by oxidized low density cholesterol (ox-LDL) is regarded as an initial step in the pathogenesis of atherosclerosis plaque formation. It is known that ox-LDL acts via binding to a number of scavenger receptors, such as SR-A1, SR-A2 and lectin-like oxidized low-density lipoprotein receptor (LOX-1). LOX-1 facilitates the uptake of ox-LDL, induces endothelial dysfunction and mediates numerous ox-LDL-induced proatherogenic effects, resulting in ox-LDL accumulation in the vessel wall [1]. LOX-1 is the main ox-LDL receptor of endothelial cells. Ox-LDL also regulates some other receptors, especially inflammatory receptors such as Toll-like receptors (TLRs) in nuclear cells. In diabetes mellitus, the effect of ox-LDL on the inflammatory receptors is still interesting.

TLRs, pathogen pattern recognition receptors, are characterized by the expression and release of cytokines and chemokines which is implicated in the development and progression of atherosclerosis. Scavenger receptors and TLRs cooperate in response to danger signals to adjust the host immune response [2]. TLR2 has a central role in innate immunity and inflammation [3]. Ox-LDL induced TLR2 and TLR4 expression at mRNA level and caused a significant activation of NF-KB in monocytes [4, 5]. TLRs
TLR2 doesn’t change in HUVECs by ox-LDL

are involved in the LPS/PGN-mediated inflammatory responses in endothelial cells [6], and it could be also involved in the inflammation induced by ox-LDL. The advanced glycation end-product of low-density-lipoprotein activates the TLR4 pathway implications for diabetic atherosclerosis [7]. TLRs activation and ligands are found to be increasing in recently diagnosed type 2 diabetic subjects [8]. We also found that TLR2/4 activation enhances endothelial inflammation in type 1 diabetes [6]. So we want to know the effect of ox-LDL on TLR2 pathway in endothelial cells, especially in diabetic condition. TLR2 expression is enhanced by LPS in HUVECs under high glucose condition [9]. It has not been determined whether TLR2 is enhanced in the inflammation induced by ox-LDL in human umbilical vein endothelial cells (HUVECs).

We wanted to study the role of TLR2 plays in the inflammatory response induced by ox-LDL in HUVECs under high glucose condition. To test this, we treated HUVECs with ox-LDL under high glucose conditions in vitro. The high glucose condition is modeling as diabetic condition in vivo [9]. Under high glucose condition, the purposes of this study are to determine: 1) the effect of ox-LDL on the inflammatory responses in HUVECs, 2) whether TLR2 levels are increased by different concentration of ox-LDL, and 3) whether the change of TLR2 level could alter the inflammation in HUVECs.

Materials and methods

Materials

HUVECs were obtained from American type culture collection (ATCC). HUVECs were cultured in endothelial cell medium (25 ml of fetal bovine serum at 5%, 5 ml of endothelial cell growth supplement (EBM-2, contains 2% FBS) (Lonza, Boulder, CO, USA) at 1% and 5 ml of penicillin/streptomycin solution at 1% was added into 500 ml) were from Scien Cell Research Laboratories (San Diego, California, USA); Ox-LDL (oxidized using CuSO4 (oxidant) in PBS) were obtained from Yiyuan Biotech (Guangzhou, Guangdong, China); TLR2 Antibody was obtained from Santa Cruz Biotechnology (Dallas, Texas, USA); neutralizing anti-TLR2 antibody (T2.5) was purchased from Invivogen (San Diego, California, USA) and ICAM1 was from Abcam (Cambridge, MA, USA); GAPDH antibody and HRP-conjugated goat anti-rabbit IgG was purchased from PTG (Pro Teintech Group, USA); IL-6 and IL-8 ELISA kits were purchased from Multi Sciences (Hangzhou, Zhejiang, China). E coli LPS were purchased from Sigma-Fluka (St. Louis, MO, USA).

Ethics statement

Our experiment was approved by the Human Ethics Committee of First Affiliated Hospital of Shantou University Medical College.

Cell culture and treatment

Cells were grown in endothelial cell growth medium. The cultures were maintained in a humidified 37°C incubator with 5% CO2. Subcultures were performed with 0.25% trypsin and 0.01% EDTA when 80% confluent. Medium was refreshed every two days.

For the experiments, cells were seeded in 500 µl complete medium in 12-well plates. After growing to confluence, medium was changed completely. Ox-LDL and LPS were diluted in complete cell culture medium containing different concentration of glucose (5.5 and 25 mmol/L) and added to the cells as normal and high glucose conditions [9]. The final concentration of LPS was 200 ng/ml, and ox-LDL was 20 µg/ml or 40 µg/ml respectively. In additional experiments, neutralizing anti-TLR2 antibody (T2.5) was added to the cells 1 h prior to adding LPS or ox-LDL. The purpose of LPS is to control the inflammatory response. The treatment time is 24 hours.

Immunoblotting

Immunoblotting was used to detect ICAM-1, TLR2, and GAPDH. After treatment, HUVECs were washed three times with cold PBS, and then lysed with adequate PBS buffer. Samples were separated on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were blocked for 1 h at room temperature with 5% dry milk in TBPS (PBS containing 0.1% Tween 20), and then incubated with the appropriate primary antibodies (ICAM-1 antibody was diluted 1:1000, TLR2 1:200, and GAPDH 1:500) overnight at 4°C. After washing with TBPS, membranes were incubated with horseradish peroxidase (HRP)-linked secondary antibodies (1:5000 dilution.
TLR2 doesn’t change in HUVECs by ox-LDL

Cytokine ELISA

Cytokine concentrations of IL-6 and IL-8 in cell culture supernatants were quantified by ELISA kits as previously reported [6]. Recombinant cytokines were used to construct standard curves. Absorbance of standards and samples was determined spectrophotometrically at 450 nm using a microplate reader (KHB lab system wallscan k3, Thermo Scientific, Finland). Results were plotted against the standard curve. The assays were carried out according to the protocols provided by the manufacturer.

Data are expressed as mean ± standard error of mean (SEM). Analysis of variance (ANOVA) was performed, and differences were considered significant when \( P<0.05 \), as verified by Fisher post-hoc test.

Results

ox-LDL induced inflammation in HUVECs

After 24 h treatment with LPS (200 ng/ml) or ox-LDL (20, 40 µg/ml), we tested IL-6, 8 expression by ELISA and ICAM-1 expression by westernblot. Stimulation with both LPS and ox-LDL in HUVECs significantly increase ICAM-1 and IL-6, 8 expression compared with those of control in both normal and high glucose condition (Figure 1, Table 1, \( P<0.05 \)). Higher concentration of ox-LDL could result in more IL-6 and IL-8 expression compared with those under normal glucose condition (Table 1, \( P<0.05 \)).
TLR2 expression didn’t change under treatment with ox-LDL in both conditions

We measured TLR2 expression in HUVECs after treatment with LPS or ox-LDL in both glucose conditions (Figure 2, P>0.05). No significant change in TLR2 expression was found compared to that in control group under both conditions, indicating TLR2 may be not involved in the inflammatory response.

TLR2 knock out could not change the inflammation induced by ox-LDL

To confirm whether TLR2 is involved in the inflammatory response induced by ox-LDL, we pretreated with TLR2 antibody 1 h before 24 h LPS or ox-LDL treatment in both conditions. Then we tested IL-6, 8 expression by ELISA and ICAM-1 expression by western blot. We didn’t find any difference in expression levels of these inflammatory cytokines and chemokines in both conditions (Figure 3; Table 2, P>0.05). If compared to those without TLR2 inhibition, this inflammation was not changed either (Tables 1 and 2, P>0.05). This result confirms that TLR2 expression was not changed accompanied by inflammatory responses under these two conditions. IL-6, 8 expression is still higher in high glucose or when it is exposed to higher concentration (Table 2, P<0.05).

Discussion

In this study, we demonstrated that ox-LDL induced inflammatory response in HUVECs under normal and high glucose conditions, were not associated with TLR2 protein expression change. Inflammatory response stimulated by LPS or ox-LDL was enhanced in high glucose conditions with or without TLR2. When exposed to higher concentration of ox-LDL, it enhanced inflammatory response in HUVECs in both glucose conditions.

It is already well known that ox-LDL plays a key role in the development of atherosclerosis, including endothelial dysfunction, macrophage invasion, and foam cell formation, etc [1]. Innate immune responses in endothelial cells are key events in vascular inflammation and the development of atherosclerosis, and is enhanced in Diabetes millitus [6, 8, 9]. Previous studies have demonstrated that ox-LDL could induce inflammatory responses in coronary endothelial cells and monocytes [10-13]. In our study, we also confirmed that ox-LDL activates the expression of adhesion molecule (ICAM-1),
TLR2 doesn’t change in HUVECs by ox-LDL

**Figure 3.** Inhibition of TLR2 level doesn’t alter ICAM-1 protein expression. HUVECs were pretreated with neutralizing anti-TLR2 antibody for 1 h, then stimulated with ox-LDL (20, 40 μg/ml) or LPS (200 ng/ml) for 24 h, and ICAM-1 protein levels were analyzed by immunoblotting. Higher ICAM-1 protein expresses in LPS and ox-LDL group than that in control (*P<0.05, compared with control, n=5).

**Table 2.** Inhibition of TLR2 level doesn’t alter IL-6 and IL-8 peptide expression

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LPS</th>
<th>20 μg/ml ox-LDL</th>
<th>40 μg/ml ox-LDL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL6 (pg/ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NG</td>
<td>307.2±34.2</td>
<td>2223.8±319.8*</td>
<td>2637.5±315.6*</td>
<td>3579.8±335.2*</td>
</tr>
<tr>
<td>HG</td>
<td>245.7±34.2</td>
<td>3998.3±231.2*</td>
<td>3549.6±175.3*</td>
<td>4486.5±196.7*</td>
</tr>
<tr>
<td><strong>IL8 (pg/ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NG</td>
<td>158.2±14.3</td>
<td>2268.0±198.4*</td>
<td>2078.5±201.3*</td>
<td>3377.2±241.9*</td>
</tr>
<tr>
<td>HG</td>
<td>186.2±19.3</td>
<td>3968.1±133.5*</td>
<td>3309.7±185.3*</td>
<td>4178.2±211.4*</td>
</tr>
</tbody>
</table>

HUVECs were pretreated with neutralizing anti-TLR2 antibody for 1 h, then stimulated with LPS (200 ng/ml) or ox-LDL (20, 40 μg/ml) for 24 h, and IL-6 and IL-8 were detected by ELISA. Ox-LDL still increased IL-6 or IL-8 expression with or without high glucose condition (*P<0.05, compared with control, n=5). More IL-6 and IL-8 expression in all groups was found under high glucose condition compared to those in normal (*P<0.05, compared with normal glucose condition, n=5).

In our study, inflammatory response didn’t decrease when we pretreated with TLR2 antibody. TLRs recognize pathogen-associated molecular patterns to initiate an innate immune response, and high TLR2 expression level on monocytes may be an independent risk factor for atherogenesis [17]. TLR2/4, in concert with scavenger receptors, could regulate the inflammatory microenvironment in atherosclerosis. TLR2 ligand peptidoglycan (PGN) and TLR4 ligand LPS have been found in vessels with early atherosclerotic lesions [18, 19]. That ox-LDL mediated endothelial dysfunction is often an initiate step for atherosclerosis formation. So we are interested in whether TLR2 is involved in endothelial dysfunction resulted from ox-LDL injury. Interestingly, we found that inflammatory response induced by ox-LDL is independent on TLR2 expression change. In murine and human lipid-rich atherosclerotic plaques, ox-LDL could regulate TLR4 expression, not TLR2 [20]. Some studies found that ox-LDL could regulate TLR2

cytokine (IL-6) and chemokine (IL-8) in HUVECs when it is exposed to normal and high glucose. Higher concentration of ox-LDL could enhance inflammatory response under stimulation by both LPS and ox-LDL. It is also found that hyperglycemia can induce endothelial dysfunction based on the overproduction of reactive oxygen species (ROS). Large clinical trials in DM have shown that hyperglycemia plays a big part in the pathogenesis of microvascular complications which is a major causal factor in the development of endothelial dysfunction and endothelial cell apoptosis [8, 14, 15]. Therefore inflammation should be enhanced under high glucose conditions [16].

expression in monocytes and macrophage [4], which also plays important roles on atherosclerosis formation. TLR2 is still involved in the atherosclerosis formation, but may just be activated by ox-LDL in monocytes, not in endothelial cells.

LOX-1 receptor is regard as a main regulator of ox-LDL, when saturation is up-regulated, it is dependent on ox-LDL concentration (10-40 μg/ml). Interestingly, LOX-1 is also decreased by a higher concentration of ox-LDL (100 μg/ml) [21]. Therefore, LOX-1 is still working at these two concentrations in our study. There is no report about crosstalk between LOX-1 and TLR2 yet. So we speculate negative change in our study should be due to the effect of LOX-1 regulation. This study also confirms there should be no crosstalk between LOX-1 and TLR2. In order to confirm the crosstalk, between LOX-1 and TLR2, a LOX-1 receptor knock out experiment would be performed in the future study.

In conclusion, the results of our study show ox-LDL induces inflammation response, and enhanced in high glucose levels, but 1) cellular TLR2 protein level is not associated with that; or 2) TLR2 knock out could not change this response. Our study suggests that TLR2 is not involved in the inflammation response induced by ox-LDL contributing to atherosclerosis in diabetes patients.

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Disclosure of conflict of interest

None.

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TLR2 doesn’t change in HUVECs by ox-LDL


