Int J Clin Exp Med 2015;8(11):22004-22010
www.ijcem.com /ISSN:1940-5901/IJCEM0013784

Original Article

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

Jilin Li[1], Shuying Chen[1*], Xiangna Cai[3], Huaiwen Wang[2], Xin Wang[1], Wei Wang[2]

Departments of [1]Interventional cardiology, [3]Plastic Surgeon, First Affiliated Hospital of Shantou University Medical College, Shantou, Guangdong Province, China; [2]Department of Cardiology, Second Affiliated Hospital of Shantou University Medical College, Shantou, Guangdong Province, China. [*]Equal contributors as first author.

Received August 1, 2015; Accepted November 10, 2015; Epub November 15, 2015; Published November 30, 2015

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 millitus 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 millitus, 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

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 Cu_2SO_4 (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% CO_2. 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 TPBS (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 TPBS, membranes were incubated with horseradish peroxidase (HRP)-linked secondary antibodies (1:5000 dilution

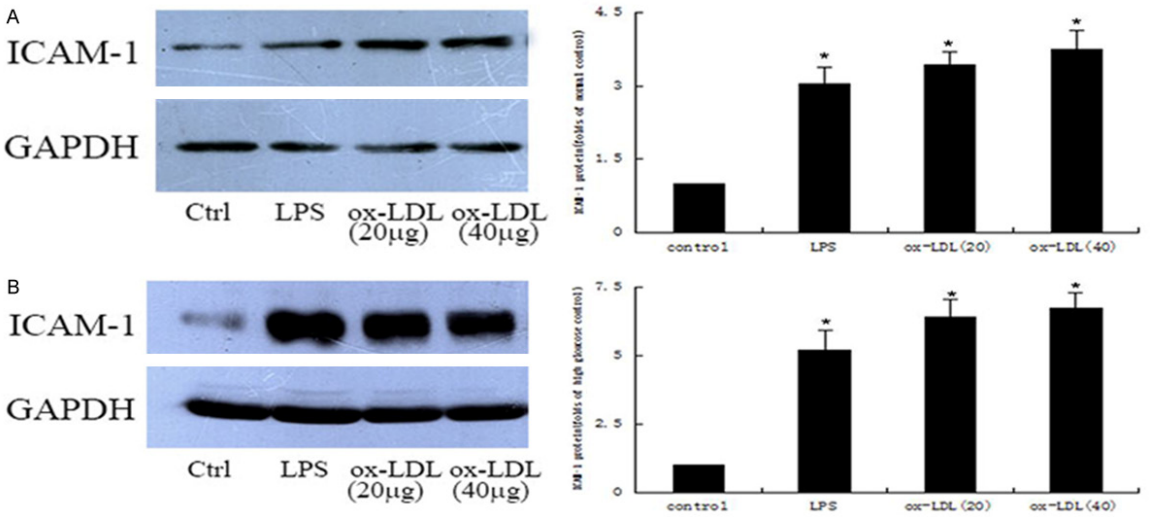

Figure 1. Ox-LDL and LPS increase ICAM-1 protein expression under both glucose conditions. HUVECs were stimulated with ox-LDL (20, 40 μg/ml) or LPS (200 ng/ml) for 24 h, and ICAM-1 protein levels were analyzed by immunoblotting. Higher ICAM-1 protein expresses in LPS and ox-LDL group than that in control (*$P<0.05$, compared with control, n=5).

Table 1. Ox-LDL enhanced IL-6 and IL-8 peptide expression mediated by ox-LDL under both glucose conditions

IL6 (pg/ml)	Control	LPS	20 μg/ml ox-LDL	40 μg/ml ox-LDL
NG	207.2±28.3	2423.9±376.0*	2443.2±305.9*	3737.0±364.8*
HG	213.2±32.7	3190.3±348.2*,#	3244.8±195.2*,#	4312.4±230.2*,#
IL8 (pg/ml)	Control	LPS	20 μg/ml ox-LDL	40 μg/ml ox-LDL
NG	143.5±11.8	2268.0±260.8*	2213.1±139.0*	3385.2±255.6*
HG	137.7±13.6	3968.1±224.1*,#	3557.2±177.0*,#	4794.3±147.3*,#

HUVECs were 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. More IL-6 and IL-8 peptide expression exhibited after stimulation with ox-LDL with or without high glucose condition (*$P<0.05$, compared with control, n=5). Lower IL-6 and IL-8 expression in all groups were found under normal glucose condition compared to those in high glucose condition (#$P<0.05$, compared with normal glucose condition, n=5).

with TPBS containing 5% dry milk) at room temperature for 1 h. Bands were developed using ECL and exposed to X-ray films. Band density was analyzed using NIH ImageJ software.

Cytokines 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 labsystem 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.

Statistic analysis

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 μg/ml or 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 medium (**Figure 1**; **Table 1**, $P<0.05$). Higher concentration of ox-LDL could result in more IL-6 and IL-8 expression (**Table 1**, $P<0.05$). Expression of IL-6, 8 was higher under high glucose if compared with those under normal glucose condition (**Table 1**, $P<0.05$).

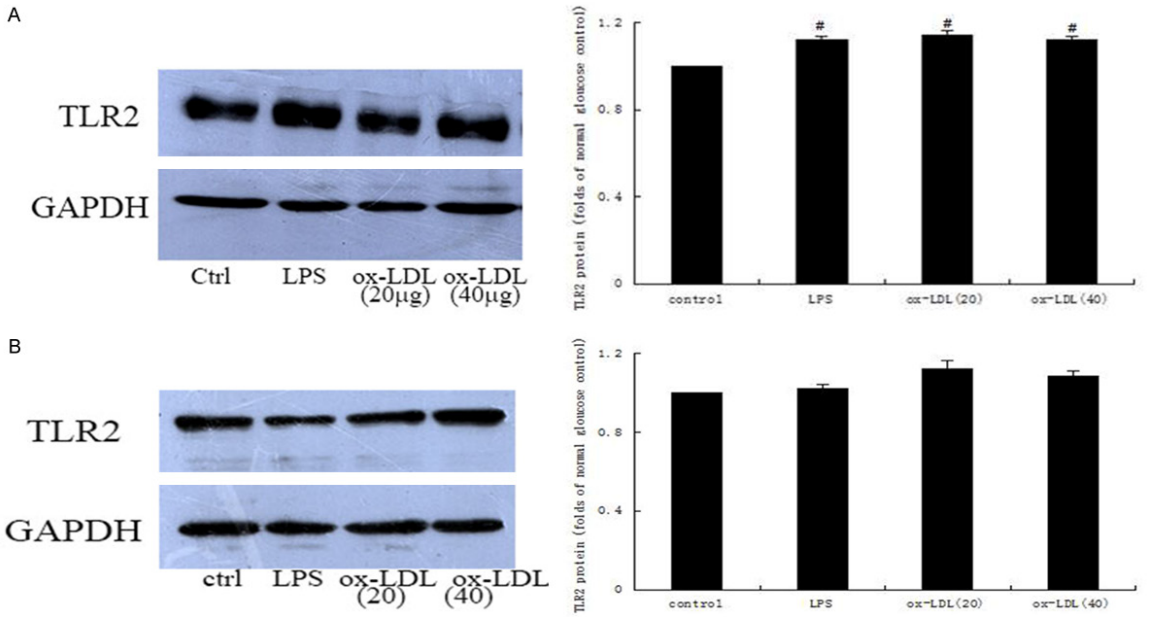

Figure 2. Induction of TLR2 expression is not associated with inflammatory response under high glucose condition. (A and B) HUVECs were stimulated with LPS (200 ng/ml) or ox-LDL (20, 40 μg/ml) for 24 h, and TLR2 protein levels were analyzed by immunoblotting. TLR2 protein level didn't change under normal (A) and high (B) glucose condition (*$P>0.05$, compared with control, n=5).

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 westernblot. 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),

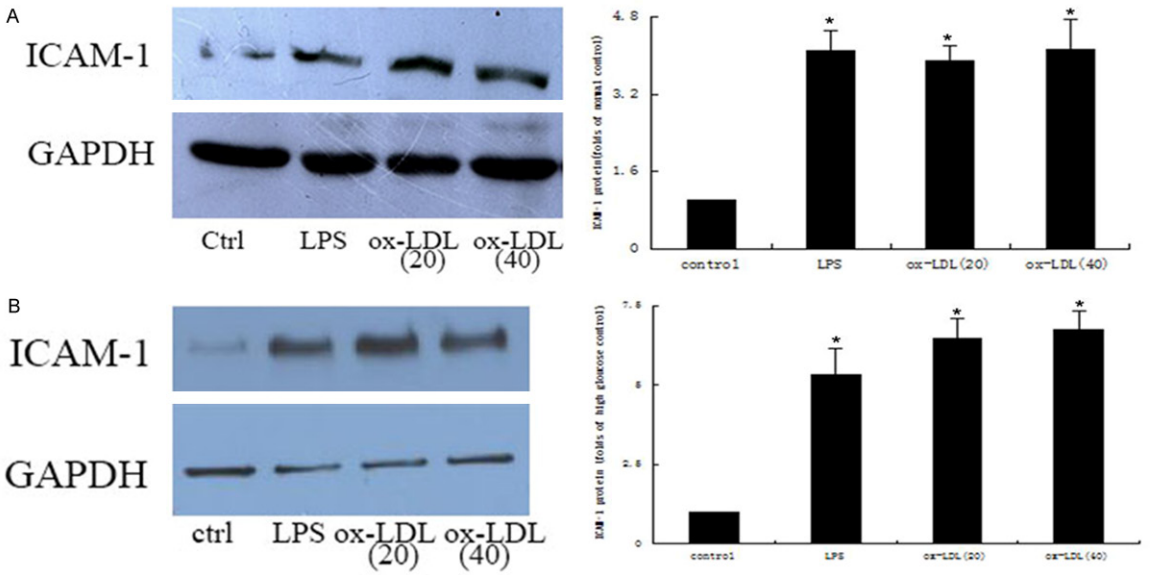

Figure 3. Inhibition of TLR2 level doesn't alter ICAM-1 protein expression. HUVECs were pretreated with neutralizing anti-TLR2 antibody for 1 h, then stimulated with ox-LDL (20, 40 μg/ml) or LPS (200 ng/ml) for 24 h, and ICAM-1 protein levels were analyzed by immunoblotting. Higher ICAM-1 protein expresses 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

IL6 (pg/ml)	Control	LPS	20 μg/ml ox-LDL	40 μg/ml ox-LDL
NG	307.2±34.2	2223.8±319.8*	2637.5±315.6*	3579.8±335.2*
HG	245.7±34.9	3998.3±231.2*,#	3549.6±175.3*,#	4486.5±196.7*,#
IL8 (pg/ml)	Control	LPS	20 μg/ml ox-LDL	40 μg/ml ox-LDL
NG	158.2±14.3	2268.0±198.4*	2078.5±201.3*	3377.2±241.9*
HG	186.2±19.3	3968.1±133.5*,#	3309.7±185.3*,#	4178.2±211.4*,#

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).

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 base 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].

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

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.

Acknowledgements

This work was supported in part by National Nature Science Foundation of China 81300123.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xiangna Cai, Department of Plastic Surgeon, First Affiliated Hospital of Shantou University Medical College, No. 57, Changping Road, Shantou, Guangdong Province, China. Tel: 754-88905462; Fax: 754-88259850; E-mail: caixiangna@126.com

References

[1] Renie G, Maingrette F, Li L. Diabetic vasculopathy and the lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1). Curr Diabetes Rev 2007; 3: 103-110.

[2] Dieudonne A, Torres D, Blanchard S, Taront S, Jeannin P, Delneste Y, Pichavant M, Trottein F, Gosset P. Scavenger receptors in human airway epithelial cells: role in response to double-stranded RNA. PLoS One 2012; 7: e41952.

[3] Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol 2001; 2: 675-680.

[4] Bhaskar S, Shalini V, Helen A. Quercetin regulates oxidized LDL induced inflammatory changes in human PBMCs by modulating the TLR-NF-kappaB signaling pathway. Immunobiology 2011; 216: 367-373.

[5] Kannan Y, Sundaram K, Aluganti Narasimhulu C, Parthasarathy S, Wewers MD. Oxidatively modified low density lipoprotein (LDL) inhibits TLR2 and TLR4 cytokine responses in human monocytes but not in macrophages. J Biol Chem 2012; 287: 23479-23488.

[6] Hodgkinson CP, Laxton RC, Patel K, Ye S. Advanced glycation end-product of low density lipoprotein activates the toll-like 4 receptor pathway implications for diabetic atherosclerosis. Arterioscler Thromb Vasc Biol 2008; 28: 2275-2281.

[7] Dasu MR, Devaraj S, Park S, Jialal I. Increased toll-like receptor (TLR) activation and TLR ligands in recently diagnosed type 2 diabetic subjects. Diabetes Care 2010; 33: 861-868.

[8] Li J, Jin C, Cleveland JC Jr, Ao L, Xu D, Fullerton DA, Meng X. Enhanced inflammatory responses to toll-like receptor 2/4 stimulation in type 1 diabetic coronary artery endothelial cells: the effect of insulin. Cardiovasc Diabetol 2010; 9: 90.

[9] Chen YY, Chen J, Hu JW, Yang ZL, Shen YL. Enhancement of lipopolysaccharide-induced toll-like receptor 2 expression and inflammatory cytokine secretion in HUVECs under high glucose conditions. Life Sci 2013; 92: 582-588.

[10] Schalkwijk CG, Stehouwer CD. Vascular complications in diabetes mellitus: the role of endothelial dysfunction. Clin Sci (Lond) 2005; 109: 143-159.

[11] van den Oever IA, Raterman HG, Nurmohamed MT, Simsek S. Endothelial dysfunction, inflammation, and apoptosis in diabetes mellitus. Mediators Inflamm 2010; 2010: 792393.

[12] Pirillo A, Reduzzi A, Ferri N, Kuhn H, Corsini A, Catapano AL. Upregulation of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) by 15-lipoxygenase-modified LDL in endothelial cells. Atherosclerosis 2011; 214: 331-337.

[13] Yu X, Xing C, Pan Y, Ma H, Zhang J, Li W. IGF-1 alleviates ox-LDL-induced inflammation via reducing HMGB1 release in HAECs. Acta Biochim Et Biophys Sin (Shanghai) 2012; 44: 746-751.

[14] Tew JG, El Shikh ME, El Sayed RM, Schenkein HA. Dendritic cells, antibodies reactive with ox-

LDL, and inflammation. J Dent Res 2012; 91: 8-16.
[15] Lappalainen J, Lindstedt KA, Oksjoki R, Kovanen PT. OxLDL-IgG immune complexes induce expression and secretion of proatherogenic cytokines by cultured human mast cells. Atherosclerosis 2011; 214: 357-363.
[16] Mehta JL, Li DY. Identification and autoregulation of receptor for OX-LDL in cultured human coronary artery endothelial cells. Biochem Biophys Res Commun 1998; 248: 511-514.
[17] Kuwahata S, Fujita S, Orihara K, Hamasaki S, Oba R, Hirai H, Nagata K, Ishida S, Kataoka T, Oketani N, Ichiki H, Iriki Y, Saihara K, Okui H, Ninomiya Y, Tei C. High expression level of Toll-like receptor 2 on monocytes is an important risk factor for arteriosclerotic disease. Atherosclerosis 2010; 209: 248-254.
[18] Burleigh ME, Babaev VR, Yancey PG, Major AS, McCaleb JL, Oates JA, Morrow JD, Fazio S, Linton MF. Cyclooxygenase-2 promotes early atherosclerotic lesion formation in ApoE-deficient and C57BL/6 mice. J Mol Cell Cardiol 2005; 39: 443-452.
[19] Nakagawa H, Tsunooka N, Yamamoto Y, Yoshida M, Nakata T, Kawachi K. Intestinal ischemia/reperfusion-induced bacterial translocation and lung injury in atherosclerotic rats with hypoadiponectinemia. Surgery 2009; 145: 48-56.
[20] Xu XH, Shah PK, Faure E, Equils O, Thomas L, Fishbein MC, Luthringer D, Xu XP, Rajavashisth TB, Yano J, Kaul S, Arditi M. Toll-like receptor-4 is expressed by macrophages in murine and human lipid-rich atherosclerotic plaques and upregulated by oxidized LDL. Circulation 2001; 104: 3103-3108.
[21] The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. N Engl J Med 1993; 329: 977-986.