Effect of oxidized low-density lipoprotein on the expression of the prorenin receptor in human aortic smooth muscle cells

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Abstract. The activation of the (pro)renin receptor (PRR) may be potentially active in the development of atherosclerotic plaques independent of angiotensin II. Our previous studies demonstrated that high glucose was able to induce the activation of PRR. The present study was designed to determine the function of oxidized low-density lipoprotein (ox-LDL) on the expression of PRR in human aortic smooth muscle cells (HASMCs). Immunofluorescence revealed that PRR was expressed in HASMCs. HASMCs were cultured with 100 μ g/ml ox-LDL for 1, 2, 4, 6, 12 and 24 h, respectively. Subsequently, HASMCs were cultured with 25, 50, 100, 150, 200 and 300 μ g/ml ox-LDL for 6 h, respectively. Reverse transcription-quantitative polymerase chain reaction and western blot analysis revealed that the expression of PRR was markedly upregulated in a time- and concentration-dependent manner, which peaked at 6 h and 50 μ g/ml, then slowly decreased. Therefore, PRR may contribute to the atherogenesis effect induced by ox-LDL.

Introduction

Atherosclerosis and cardiovascular disease remain one of the major causes of mortality worldwide. Ox-LDL has a central proatherogenic role in the arterial wall (1-3). It has a multitude of actions on vascular smooth muscle cells, including inducing their migration and proliferation as well as altering their phenotype to foam cells (4-6). Ox-LDL also results in the generation of reactive oxygen species (ROS) from vascular smooth muscle cells (7). Ox-LDL, through increasing ROS production, leads to an increase in the generation of a variety

of growth factors, including fibroblast growth factor (8,9), insulin-like growth factor-1 (10) and epidermal growth factor (11) as well as expression of their receptors, therefore inducing vascular smooth muscle cell proliferation and hypertrophy. Ox-LDL is also able to induce apoptosis in smooth muscle cells (12).

The (pro)renin receptor (PRR) constitutes a novel component of the renin-angiotensin system (RAS) and has attracted significant attention in previous years due to its versatile functions (13). Numerous studies have verified that when the renin precursor binds to its receptor, it directly triggers angiotensin II independent reactions, which may be potentially active in the development of atherosclerotic plaques (14,15). The binding to PRR and the ability to induce a signal transduction cascade independent of the generation of angiotensin II (13,16), including the activation of mitogen-activated protein kinase (MAPK) (17) and enhancement of the phosphorylation of extracellular signal-regulated kinase (ERK1/2) (18), promotes fibrosis gene expression, including transforming growth factor- β , plasminogen activator inhibitor-1, fibronectin and collagen proteins (19,20). However, to the best of our knowledge, there is little data demonstrating the direct effect of the atherogenic condition on the PRR.

A previous study by our group demonstrated that the conditions of atherogenesis, including high glucose and high blood lipids were able to upregulate the expression of PRR in cultured human umbilical vein endothelial cells (21). Therefore, cultured human aortic smooth muscle cells (HASMCs) were utilized to investigate the effect of ox-LDL on the expression of PRR.

Materials and methods

Cell culture. HASMCs were obtained from Lifeline Cell Technology (Beijing, China) and cultured according to the manufacturer's instructions. The cells were cultured in Vasculife SMC cell culture medium, containing Vasculife basal medium and LifeFactors SMC (cat no. LS-1040; Lifeline Cell Technology) at 37°C in an atmosphere of 95% air and 5% CO₂ according to the manufacturer's instructions. Cells were seeded in six-well plates and the culture medium was changed daily. Prior to each experimental treatment, cells were serum starved for 24 h under serum-free conditions. The concentration of human ox-LDL (24.5 nmoles of MDA/mg protein; Qingdao Haicon Biotechnology Co., Ltd., Qingdao,

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China) was primarily based on a previously published study demonstrating their effectiveness in vascular smooth muscle cells (21). According to these results, 100 $\mu\text{g/ml}$ ox-LDL was used to stimulate HASMCs for different time periods, which were divided into 1, 2, 4, 6, 12 and 24 h subgroups. Subsequently, the strongest expression time point of PRR was selected and cells were treated with different concentrations of ox-LDL, which were divided into 25, 50, 100, 150, 200 and 300 $\mu\text{g/ml}$ subgroups. At the end of each experiment, cells were harvested for the preparation of whole-cell lysates and total RNA extraction.

Immunofluorescence. The expression of PRR in HASMCs was detected using immunofluorescence staining. HASMCs were seeded at a density of 5×10^4 cells/ml into 24-well plates, cultivated and divided into groups. At the end of culture, the HASMCs were fixed with paraformaldehyde for 30 min. Cells were incubated with the primary antibody (polyclonal rabbit anti-human ATP6IP2 antibody; 1:700 dilution; ab64975; Abcam, Cambridge, MA, USA) at 4°C overnight. Following incubation with the fluorescein-labeled secondary antibody (fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G; 1:200; sc-2012, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 37°C for 40 min, HASMCs were observed using fluorescence microscopy (DM4000 B LED; Leica, Mannheim, Germany) and images were immediately captured. The same process without the primary antibody and secondary antibody was used as a negative control. Green fluorescence of the cell membrane indicated positive expression of PRR.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted immediately from cultured cells using TRIzol reagent (Gibco-BRL, Dalian, China) according to the manufacturer's instructions. Total RNA was reverse transcribed using the Prime Script RT reagent kit with gDNA Eraser (Perfect Real Time; Takara Bio Inc., Dalian, China). RT-qPCR was performed using SYBR Premix Ex Taq™ II (Takara Bio Inc.) with the Roche LightCycler® 480 Sequence Detection System (Roche Diagnostics GmbH, Mannheim, Germany). Samples were run in triplicate in separate tubes to permit quantification of the target gene normalized to GAPDH, which was used for equal loading. Primer sequences are shown in Table I. The PCR amplification program was as follows: 95°C for 30 sec, 95°C for 5 sec and 60°C for 20 sec for 40 cycles.

Western blot analysis. Cells were lysed in 100 μl of lysis buffer (Nanjing KeyGen Biotech. Co., Ltd., Nanjing, China). Protein concentrations were measured using a protein assay kit (Nanjing KeyGen Biotech. Co., Ltd.). The protein (25 μg) obtained from each lysate was electrophoresed on a 15% SDS-polyacrylamide gel (90 mV, 30 min; 130 mV, 90 min) and transferred onto a nitrocellulose membrane (Merck Millipore, Darmstadt, Germany) using a Mini-PROTEAN 3 system (120 mV, 1.5 h; Bio-Rad, Hercules, CA, USA). The membrane was incubated overnight with polyclonal rabbit anti-human ATP6AP2 antibody (1:700 dilution; Abcam) and polyclonal rabbit anti-human β -actin (1:500 dilution; bs-0061R; Bioss, Beijing, China). The specific binding

Table I. Primers used for reverse transcription-quantitative polymerase chain reaction.

Gene	Sequence (5'-3')
ATP6AP2 (NM_005765)	
Forward	TGGAAATTGGCCTATACCAGGAG
Reverse	GTAGCCCGAGGACGATGAAAC
GAPDH (NM_002046)	
Forward	GCACCGTCAAGGCTGAGAAC
Reverse	TGGTGAAGACGCCAGTGGA

was detected using horseradish peroxidase-labeled goat anti-rabbit IgG (1:7,500; ZSGB-BIO, Beijing, China) and an enhanced chemiluminescence detection kit (Perkin-Elmer, Waltham, MA, USA). The bands were quantified using Image Pro-Plus 5.0 software (Media Cybernetics, Rockville, MD, USA).

Statistical analysis. Standard statistical methods from the SPSS statistical analysis system 16.0 (SPSS, Inc., Chicago, IL, USA) were used. Statistical comparisons were made using a two-way analysis of variance. Data are presented as the mean $\pm$ standard error of the mean. $P < 0.05$ was considered to indicate a statistically significant difference. Duplicate wells were analyzed for each experiment and each experiment was performed independently at least three times.

Results

PRR expression in HASMCs. Immunofluorescence techniques verified that PRR was expressed in HASMCs (Fig. 1), indicating that PRR was abundant in HASMCs and mainly present in the cell membrane and cytoplasm.

Effect of ox-LDL on the expression of PRR at different time periods. HASMCs were incubated with a final concentration of 100 $\mu\text{g/ml}$ ox-LDL for 0, 1, 2, 4, 6, 12 and 24 h. It was found that the expression of PRR was significantly upregulated by ox-LDL in a time-dependent manner (Fig. 2). The mRNA and protein expression of PRR began to increase 2 h following incubation and reached a peak level at 6 h and then decreased moderately, however, maintaining a higher level of expression than the control group at 24 h.

Effect of ox-LDL on the expression of PRR at different concentrations. Based on the above results, HASMCs were incubated with 0, 25, 50, 100, 150, 200 and 300 $\mu\text{g/ml}$ ox-LDL for 6 h, respectively. Compared with the control group, the expression of PRR mRNA increased in the group treated with 25 $\mu\text{g/ml}$, reached a peak in the 50 $\mu\text{g/ml}$ group, then decreased moderately in the remaining groups (Fig. 3A), revealing a concentration-dependent effect. The expression of PRR protein was similar to that of the mRNA, however, maintained significantly higher levels than the control group following decrease (Fig. 3B).

Figure 1. Expression of the (pro)renin receptor in human aortic smooth muscle cells. Nuclear staining with Hoechst. (A) Staining for (pro)renin receptor (green fluorescence). (B) Negative control (magnification, x200).

Figure 2. (A) Expression of the PRR in human aortic smooth muscle cells stimulated by 100 μ g/ml ox-LDL for different time periods detected by reverse transcription-quantitative polymerase chain reaction and (B) western blot analysis. * $P < 0.05$ vs. the control group. PRR, (pro)renin receptor.

Figure 3. (A) Expression of the PRR in human aortic smooth muscle cells stimulated by different concentrations of ox-LDL for 6 h detected by reverse transcription-quantitative polymerase chain reaction and (B) western blot analysis. * $P < 0.05$ vs. the control group. PRR, (pro)renin receptor.

Discussion

In the present study, immunofluorescence demonstrated that PRR was expressed on the cell membrane and cytoplasm in HASMCs (Fig. 1A), which is consistent with a previous study (22) and the PRR was abundant in HASMCs.

Binding of (pro)renin to the PRR increases the catalytic activity of prorenin and renin, resulting in increased RAS

activation (13). Additionally, intracellular signaling cascades, including the MAPK and ERK1/2 pathways as well as the phosphorylation of heat shock protein 27 (HSP 27) (18,23,24) are triggered, resulting in the expression of profibrotic and inflammatory molecules. These effects promote the occurrence and development of atherosclerosis.

The present study demonstrated that the expression of PRR was upregulated in a time- and concentration-dependent

manner stimulated by ox-LDL, indicating that PRR is involved in ox-LDL-induced atherosclerosis. This demonstrated the effect of ox-LDL on the expression of PRR for the first time, to the best of our knowledge.

However, the mechanisms underlying atherosclerosis formation induced by ox-LDL are varied, including via its own LOX-1 receptor, promoting the generation of ROS, promoting the formation of foam cells and promoting the phenotypic transformation of SMC.

Therefore, whether the upregulation of PRR expression was induced by ox-LDL directly remains to be elucidated. Further studies are required to determine the association between PRR and LOX-1, the receptor of ox-LDL.

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