Altered expression of α-actin, smooth muscle myosin heavy chain-1 and calponin in cultured smooth muscle cells by oxidized low density lipoproteins

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Abstract The expression of the contractile proteins, α-actin, smooth muscle myosin heavy chain-1 (SM1) and calponin present in smooth muscle cells (SMC) in the presence of oxidized low density lipoproteins (oxLDL) was investigated in two different cell cultures: the mouse smooth muscle cell line SVSC and rat smooth muscle cells (RSMC). Exposure of the cells to 187 μg protein/ml oxLDL for 24 h reduced the expression of all three contractile proteins in both cell cultures when compared to cells incubated in the presence of native LDL. This investigation of the response of SMC contractile proteins to oxLDL may provide further insights into the mechanisms by which oxidatively modified LDL is atherogenic and suggests that oxLDL may contribute to the regulation of the expression of the genes responsible for the synthesis of smooth muscle cell contractile proteins.

Key words: Oxidized low density lipoprotein; Smooth muscle cell; α-Actin; Smooth muscle myosin heavy chain-1; Calponin; Atherosclerosis

1. Introduction

It is widely asserted that oxidized low density lipoproteins (oxLDL) play a central role in the pathogenesis of atherosclerosis: a disease characterized by lesions resulting from an excessive, inflammatory-fibroproliferative response to various forms of insult to the endothelium and smooth muscle of the artery wall, ultimately leading to vessel occlusion responsible for heart attacks and strokes [1–3]. Oxidatively modified LDL is present in atherosclerotic lesions [4] and is believed to be a key event in the development of atherosclerosis, mainly due to its enhanced uptake by macrophages [5] and its ability to alter gene expression in arterial cells. In fact, oxLDL exerts multiple cellular effects in macrophages and endothelial cells that enhance lesion progression. In vascular smooth muscle cells (SMC), oxLDL has been shown to induce an array of responses, such as increased expression of intracellular antioxidant stress proteins, heme oxygenase-1 and macrophage stress protein MSP23 [6], which act as a cytoprotective defence against oxidative stress, and increased expression of heat shock protein hsp70 [7], which affords protection against proteotoxic factors. In addition, mildly oxidized LDL has been observed to evoke a sustained and intense calcium-dependent retraction of vascular SMC [8] and stimulated cell proliferation by activation of metabolic enzymes [9]. Oxidation of LDL is also associated with the stimulation of collagen production in SMC [10] and with the formation of several substances which affect the growth of SMC such as lysophosphatidylcholine which stimulates DNA synthesis [11]. Since it has been observed that SMC acquire certain phenotypic features during atheroma formation such as changes in the relative expression of actin isoforms [12], it was of interest to address the question of whether oxLDL may induce changes in the expression of SMC contractile proteins, particularly if one considers that the development of atherosclerotic plaques is characterized by the transition of the SMC phenotype from a contractile to a synthetic one. In this study, we investigated the effect of oxLDL on the expression of α-actin, the most abundant actin isoform in normal vascular SMC [13], of smooth muscle myosin heavy chain-1 (SM1), of calponin, a thin filament protein involved in the regulation of actin-myosin interactions in SMC [14], and of non-muscle myosin in two different cell cultures: the mouse smooth muscle cell line SVSC and rat smooth muscle cells (RSMC).

2. Materials and methods

2.1. Cell culture and treatment with LDL

RSMC were isolated from Wistar rat aortas by enzymatic digestion and propagated as described [15]. Passages from 10 to 14 of RSMC were used for the experiments. SVSC cells were isolated and established from aortas of transgenic mice harboring the SV40 large T-antigen gene [16]. The SVSC and RSMC cells obtained were cultured in medium 199 supplemented with 10% fetal bovine serum, 2.2 g/l $NaHCO_3$, 100 U/ml penicillin G and 100 μg/ml streptomycin, in collagen-coated plastic petri dishes (Corning) in an incubator at 33°C for the SVSC cells and at 37°C for the RSMC cells in a humidified atmosphere of 5% CO_2. For the experiments, the cells were seeded in six multiwell culture dishes at a density of 5×10^4 cells/well, and grown for 3 days in these conditions. Twenty-four hours before LDL incorporation, the medium was removed and replaced with serum-free medium 199; SVSC cells were incubated at 39°C while RSMC cells were kept at 37°C in a 5% CO_2 atmosphere. Cells were then incubated with native or oxidized LDL (187 μg protein/ml) for 24 h at 39°C and 37°C for SVSC and RSMC cells, respectively. Exposure to LDL was terminated by gently washing the cells three times with ice-cold Dulbecco's phosphate buffered saline.

2.2. Western blot analysis

Cells were lysed in buffer (1% Triton X-100, 50 mM Tris, 1 mM EDTA, 0.1 mM *p*-amidinophenylmethylsulfonyl fluoride (pAPMSF)). Sample total protein content was determined according to Lowry et al. [17] and equal amounts of protein concentration from each sample were boiled in a mixture of 10 mM Tris, 20% glycerol, 1% SDS, 0.02% bromophenol blue for 3 min. Proteins and molecular weight markers were subjected to SDS-PAGE on either 3% or 12.5% polyacrylamide gels, using the buffer solution composed of 25 mM Tris, 192 mM

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glycine, 0.1% SDS. Electrophoresed proteins were transferred onto a nitrocellulose membrane using a semi-dry blotter AE-665 (Atto, Japan) and Western blotting solution composed of 0.1 M Tris, 0.2 M glycine, 0.01% SDS, 20% methanol. The membranes were blocked in PBS/0.1% Tween-20 (PBST) containing 5% skimmed milk for 30 min at 25°C. The protein blots were then incubated with a 1:1000 dilution of either monoclonal anti-α smooth muscle actin antibody, anti-calponin antibody or anti-non-muscle myosin antibody or with a 1:20 dilution of anti-smooth muscle heavy chain-1 antibody (KM995) and incubated at 37°C for 30 min. After three washes with PBST, the membranes were incubated for 1 h at room temperature with a 1:2000 dilution of a secondary antibody conjugated to horseradish peroxidase used as the marker for the antigenically reacted proteins; these were anti-mouse IgG, anti-rabbit IgG and anti-rat IgG. The membranes were then washed five times in PBST and the immune complex was detected by an enhanced chemiluminescent method (ECL, Amersham). Densitometric analysis of the autoradiograms was carried out using an CS-9000 dual wavelength flying spot scanning densitometer (Shimadzu, Japan).

2.3. Preparation of LDL

LDL (density 1.019–1.063 g/ml) was isolated from normal human blood by ultracentrifugation in the presence of EDTA, followed by overnight dialysis against PBS at 4°C, as described previously [18]. After protein determination using the Lowry method, LDL (200 μg protein/ml) in PBS was oxidized by incubation with 5 μM $CuSO_4 \cdot 5H_2O$ at 37°C for 3 h. The reaction was stopped by addition of 1 mM EDTA followed by overnight dialysis of oxLDL against PBS at 4°C. Oxidized LDL was then passed through a 0.22 μm sterilized Millipore filter followed by protein determination of oxLDL. The oxidation and purity of sterilized LDL was evaluated by electrophoresis mobility on agarose gels and it was stored at 4°C under N_2 for no longer than 2 weeks.

2.4. Materials

Medium 199, penicillin and streptomycin were purchased from Gibco BRL, Tween-20 and anti-α smooth muscle actin antibody were purchased from Sigma, anti-calponin antibody and KM995 were obtained as described previously [19], anti-non-muscle myosin heavy chain antibody was purchased from Paesel and Lorei (Germany), Lowry reagents from Bio-Rad. Secondary antibodies, enhanced chemiluminescence Western blotting detection reagents and Hyperfilm-MP autoradiography film were purchased from Amersham International, Amersham, UK. All chemicals used were analytical grade reagents.

3. Results

In order to investigate the expression of contractile proteins in the presence of oxLDL, SVSC cells and RSMC were used.

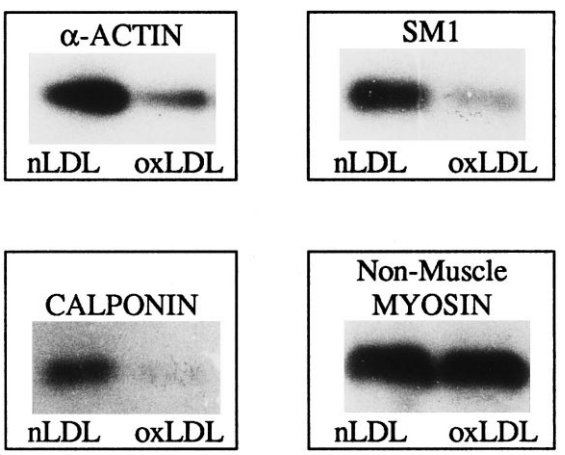

Fig. 1. Comparison of the effects of native and oxidized LDL on the expression of α-actin, SM1, calponin and non-muscle myosin by Western blot analysis. SVSC cells were incubated for 24 h in the presence of either 187 μg protein/ml native or oxLDL. Data are representative of experiments in three different cell cultures.

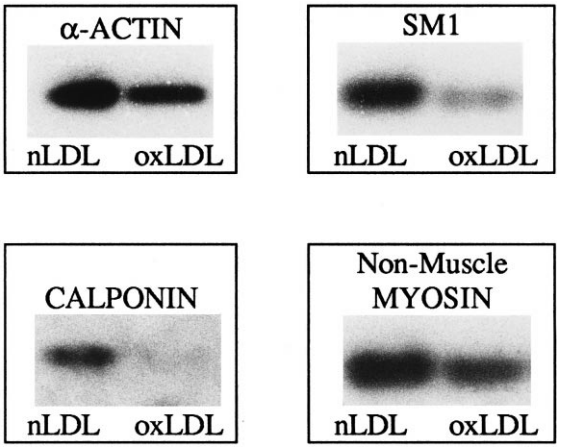

Fig. 2. Comparison of the effects of native and oxidized LDL on the expression of α-actin, SM1, calponin and non-muscle myosin by Western blot analysis. RSMC cells were incubated for 24 h in the presence of either 187 μg protein/ml native or oxLDL. Data are representative of experiments in three different cell cultures.

SVSC cells possess a unique characteristic – their cell growth is regulated by the cultivation temperature [19] while growth of RSMC is not. Fig. 1 shows the expression of α-actin, SM1, calponin and non-muscle myosin in SVSC smooth muscle cells when exposed to 187 μg protein/ml of native or oxidized LDL after a 24 h incubation period. It can be observed that there is a remarkable decrease in expression of all the smooth muscle contractile proteins in those samples incubated in the presence of oxLDL compared to native LDL. More specifically, there is a 52% decrease in α-actin expression, a 72% decrease in SM1 and a 53% decrease in calponin in these samples. However, non-muscle myosin seems to be unaffected by the presence of oxLDL. In Fig. 2, the expression of the same proteins in RSMC when exposed to 187 μg protein/ml of native or oxidized LDL after a 24 h incubation period is reported. With α-actin there is only an 18% decrease in expression in samples incubated with oxLDL compared with native LDL, while with SM1 and with calponin there is a 49% and a 46% decrease, respectively. In this cell line, oxLDL also seems to mildly affect the expression of non-muscle myosin as a 22% decrease in expression is observed.

4. Discussion

During the last few years, active expression of several genes has been reported in developing atherosclerotic lesions and oxLDL is one of the factors which contributes to the regulation of gene expression in these lesions [20,21]. In this study, we provide the first evidence that the expression of the proteins α-actin, smooth muscle myosin heavy chain-1 and calponin, involved in smooth muscle cell contraction, is altered in cultured smooth muscle cells when these are incubated in the presence of oxidatively modified LDL. The comparison between mouse SVSC and RSMC cell cultures offered some interesting information. Unlike RSMC, SVSC cells seem to respond to oxLDL to a greater extent since there is an increased downregulation in the expression of the proteins especially for α-actin. Previous studies have reported a switch in actin expression in SMC of human atheromatous plaques and in experimental rat aortic intimal thickening when compared with SMC of normal media [12]. This switch involves the ratio

of the different actin isotypes: from a predominance of the α-actin isoform in normal media to a predominance of the β form and a noticeable amount of the γ form in atheromatous plaque. It has also been reported that myosin heavy chain isoform expression changed in atherosclerotic human aorta and experimental atherosclerosis of rabbit carotid artery [22,23]. Adult rabbit smooth muscle cells contain two types of myosin heavy chain isoforms, SM1 and SM2, but not non-muscle myosin heavy chain. Interestingly, non-muscle myosin heavy chain was re-expressed in proliferating smooth muscle cells of atherosclerotic neointima. The decrease in α-actin and SM1 expression observed in this study when SMC were incubated in the presence of oxLDL could possibly be due to their switch in isoform. Therefore, it would seem from the results presented here that oxLDL could be one of the factors responsible for the decrease in α-actin and SM1 expression observed in human atheromatous plaque. However, not only does oxLDL seem to exert an influence on the expression of these contractile proteins, it also seems to influence the expression of calponin. The expression of non-muscle myosin does not seem to be greatly affected by oxLDL if only marginally in RSMC. This investigation of the response of SMC contractile proteins to oxLDL may provide further insights into the mechanisms by which oxidatively modified LDL is atherogenic. Secondly, it seems conceivable that developments in the analysis of gene expression in the artery wall at the cellular level will help in clarifying the sequence of events which lead to the cellular dysfunction and cytoskeletal alteration that accompany atherosclerotic lesions.

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