

Effect of irreversibly glycated LDL in human vascular smooth muscle cells: lipid loading, oxidative and inflammatory stress

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Abstract

The major complication of diabetes is accelerated atherosclerosis, the progression of which entails complex interactions between the modified low-density lipoproteins (LDL) and the cells of the arterial wall. Advanced glycation end product-modified-LDL (AGE-LDL) that occurs at high rate in diabetes contributes to diabetic atherosclerosis, but the underlying mechanisms are not fully understood. The aim of this study was to assess the direct effect of AGE-LDL on human vascular smooth muscle cells (hSMC) dysfunction. Cultured hSMC incubated (24 hrs) with human AGE-LDL, native LDL (nLDL) or oxidized LDL (oxLDL) were subjected to: (i) quantification of the expression of the receptors for modified LDL and AGE proteins (LRP1, CD36, RAGE) and estimation of lipid loading, (ii) determination of NADPH oxidase activity and reactive oxygen species (ROS) production and (iii) evaluation of the expression of monocyte chemoattractant protein-1 (MCP-1). The results show that exposure of hSMC to AGE-LDL (compared to nLDL) induced: (a) increased NADPH oxidase activity (30%) and ROS production (28%) by up-regulation of NOX1, NOX4, p22phox and p67phox expression, (b) accumulation of intracellular cholesteryl esters, (c) enhanced gene expression of LRP1 (160%) and CD36 (35%), and protein expression of LRP1, CD36 and RAGE, (d) increased MCP-1 gene expression (160%) and protein secretion (300%) and (e) augmented cell proliferation (30%). In conclusion, AGE-LDL activates hSMC (increasing CD36, LRP1, RAGE), inducing a pro-oxidant state (activation of NADPHox), lipid accumulation and a pro-inflammatory state (expression of MCP-1). These results may partly explain the contribution of AGE-LDL and hSMC to the accelerated atherosclerosis in diabetes.

Keywords: AGE-LDL • CD36 • glycated LDL • lipid loading • LRP1 • MCP-1 • NADPH oxidase • RAGE • SMC proliferation • vascular hSMC

Introduction

In human beings, diabetes mellitus associates with increased incidence of macro- and microvascular complications including coronary artery and peripheral vascular diseases. In diabetic-associated vascular disease, low-density lipoproteins (LDL) are modified and play a key role in the accelerated progression of atherosclerosis [1–3]. Chronic hyperglycaemia enhances glucose-induced LDL

oxidation and/or glycation, which increases its pro-atherogenic properties [4] and promotes vascular injury by a mechanism that is scantily defined. Irreversible glycation starts with the non-enzymatic addition of reducing sugars to the native LDL (nLDL) lysine residues, followed by additional reactions leading to the formation of sugar-amino acid adducts, collectively known as advanced glycation end-products (AGE). A correlation between arterial tissue AGE and circulating AGE-ApoB, and the contribution of AGE-specific receptors (RAGE) in atheroma formation was reported [5].

Smooth muscle cells (SMC) are major contributors to the initiation and early progression of the atherosclerotic plaque in human beings and animal models [6]. Within the plaque SMC migrate, proliferate, secrete chemokines that enhance the accrual

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of monocytes, and ultimately take up transcytosed, modified lipoproteins (Lp) and turn into foam cells. Like all cells of the vessel wall, SMC are exposed to AGE-LDL that accumulates in the atheroma of diabetic patients [7, 8] and animal models of diabetes [9]; the specific effects of AGE-LDL on SMC are not known.

The involvement of the oxidative stress and the NADPH oxidase complex (NADPHox) in the dysfunction of vascular cells is well established [10, 11]. It is known that oxidized LDL (oxLDL) regulates the expression of NADPHox in human endothelial cells and vascular human SMC (hSMC) [12], but there are no data on the effect of AGE-LDL on the modulation of NADPHox in hSMC.

The expression of native LDL receptors on arterial cells is tightly controlled and feedback regulated, in contrast to the uncontrolled uptake of modified LDL and subsequent foam cell formation occurring *via* alternative receptors (CD36, LRP1, scavenger receptors A and B1, and RAGE) [13]; there are no data on the effect of AGE-LDL on these receptors.

MCP-1 is a potent pro-inflammatory chemokine, whose expression in hSMC is up-regulated by high glucose, leading to increased monocyte-SMC adhesive interactions [14]. The effects of AGE-LDL on MCP-1 expression are also not known.

In this study, we provide evidence that AGE-LDL activates hSMC (inducing LRP1, CD36, RAGE), triggering a pro-oxidant state (activation of NADPHox) and pro-inflammatory state (expression of MCP-1) and lipid accumulation. To the best of our knowledge, this is the first report on the effects of AGE-LDL on vascular hSMC, providing a mechanism that may explain accelerated atherosclerosis in diabetic patients.

Materials and methods

Preparation of AGE-LDL

LDL was isolated from the plasma of healthy donors from the Blood Transfusion Center Bucharest by density gradient ultracentrifugation as previously described [15]. The LDL fraction was collected, dialysed against phosphate buffer saline (PBS), pH 7.4, 4°C in the dark, stored in sterile conditions in the presence of antioxidants (1 mg/ml EDTA and 10 µM BHT) at 4°C and used within 7 days. AGE-LDL was prepared by incubation of nLDL (2 mg protein/ml) with D(+)-glucose (0.2M final concentration), for 4 weeks, at 37°C, under sterile conditions with antioxidants (1 mg/ml EDTA and 10 µM BHT). Prior to the experiments, AGE-LDL was extensively dialysed against PBS, pH 7.4, 4°C. OxLDL was prepared by incubating nLDL under sterile conditions with 10 µM copper chloride (24 hrs, at 37°C), in the absence of antioxidant protection. The oxidative reaction was stopped by addition of 1 mg/ml EDTA and after extensive dialysis against PBS, pH 7.4, 4°C, oxLDL was stored at 4°C, under sterile conditions and used within 7 days.

Characterization of AGE-LDL

Quantification of the free, non-glycated amino groups was done using the trinitrobenzene sulphonic acid assay (TNBSA) [16]. The extent of LDL

protein glycation was determined by thiobarbituric assay (TBA) and expressed as nmoles of 5-hydroxymethylfurfuraldehyde (HMF) per mg LDL protein [17]. Protein concentration was measured by the Lowry method as modified for lipoproteins [18] employing bovine serum albumin as a standard. The formation of AGE products, pentosidine and furoyl furanyl imidazole (FFI), was monitored by spectrofluorimetry (excitation/emission 335/385 nm and 370/450 nm) [19]. The lipid peroxide level was determined as thiobarbituric acid reactive substances (TBARS) and expressed as nmol malondialdehyde (MDA)/mg protein [20].

The electric charge of AGE-LDL, oxLDL and nLDL was determined by agarose gel electrophoresis (0.6%) and expressed as the relative electrophoretic mobility (Rf). SDS-PAGE was employed to assess the degradation of LDL's apoB.

Cell culture and experimental design

Aortic hSMC were obtained by explantation from the media of foetal thoracic aorta and grown in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal calf serum (FCS) (v/v) and supplemented with non-essential amino acids and antibiotics [21]. At confluence, hSMC were starved for 24 hrs by incubation with DMEM in the presence of 0.2% FCS and then exposed to nLDL, AGE-LDL or oxLDL (100 µg protein/ml medium) for 24 hrs. As control, hSMC were cultured in the same conditions without LDL exposure. Cultured cells were used between the ninth and twelfth passages.

Lipid loading induced by AGE-LDL

The cells plated on coverslips placed in 24 well plates (5×10^4 cells/well) were incubated with AGE-LDL, nLDL or oxLDL (100 µg protein/ml) for 24 hrs. After washing with PBS, the cells were fixed in 2% paraformaldehyde (10 min.), stained (6 min.) with Nile Red (150 ng/ml PBS) to evidence the neutral lipids and examined by fluorescence microscopy with a Microphot FXA Nikon microscope using filter block B-2A ($\lambda_{ex} = 420-490$ nm, $\lambda_{em} > 520$ nm).

Quantification of free and esterified cholesterol

Total lipids from cells incubated with nLDL, AGE-LDL or oxLDL were extracted in chloroform:methanol (2:1) and the total cholesterol (TC) and free cholesterol (FC) were determined as described [22]. The fluorescence was measured using a Shimadzu spectrofluorimeter (excitation 325 nm, emission 415 nm). The cholesteryl esters (CE) content of the cells was expressed as CE/TC*100 ratio.

Evaluation of the viability, metabolic competence and proliferation rate of hSMC

Cells viability was measured as a function of the mitochondrial activity using a cell growth determination kit based on tetrazolium salt (MTT, Sigma, St. Louis, MO, USA). Evaluation of the energy metabolism of hSMC incubated with AGE-LDL, nLDL and oxLDL was done by measuring the intracellular ATP level with ViaLight Plus Kit (Lonza, Walkersville, MD, USA).

Table 1 Real-time PCR primers employed

	Forward	Reverse
28S rRNA	AAACTCTGGTGGAGGTCGGT	CTTACCAAAAGTGCCCACTA
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCGTA
LDLR	CAATGTCTCACCAAGCTCTG	TCTGTCTCGAGGGGTAGCTG
LRP1	GAGCTGAACCACGCCTTTG	GGTAGACTGCCACTCCGATAC
CD36	ATGTAACCCAGGACGCTGAG	GTCGCAGTGACTTCCCAAT
RAGE	TCCAATTGGTGGTGGAGCCAGAA	TCGCCTGGTTCGATGATGCGTAT
MCP-1	AGCATGAAAGTCTCTGCCGCCCTCTG	ATTACTTAAGGCATAATGTTTCACA
NOX1	CACAAGAAAAATCCTGGGTCAA	GACAGCAGATTGCCGACACACA
NOX4	TGGCTG CCCATCTGGTGAATG	CAGCAGCCCTCCTGAAACATGC
p67 ^{phox}	CGGTGCTGGCATCTGTGGTGG	GGGATGTCGGAC TGCGGAGAGC
p22 ^{phox}	GTTTGTGTGCCTGCTGGAGT	TGGGCGGCTGCTTGTATGGT

GAPDH: glyceraldehyde 3-phosphate dehydrogenase; LDLR: Low-density lipoprotein receptor; LRP1: LDL receptor-related protein 1; RAGE: receptor for advanced glycation end-products; MCP-1: monocyte chemotactic protein-1.

In other experiments, we have measured the hexose uptake in hSMC exposed to AGE-LDL, nLDL or oxLDL. Hexose uptake by hSMC was estimated by measuring the glucose concentration in the culture medium before and after incubation with LDL. The proliferation rate was determined by a fluorimetric assay using Hoechst 33258, which measures cellular DNA content [23].

Determination of NADPH oxidase activity

Quiescent hSMC were exposed for 24 hrs to 100 µg/ml AGE-LDL, nLDL or oxLDL. The NADPH activity was determined in cell homogenates by lucigenin-enhanced chemiluminescence assay [24] using a low concentration of lucigenin (5 µmol/l) to oxLDL minimize artifactual O₂⁻ production due to redox cycling [25]. The reaction mixture comprised 50 mM PBS, 1 mM EGTA, pH 7.0, 5 µmol/l lucigenin and 100 µmol/l NADPH. The reaction started by addition of cell homogenate (100–150 µg of protein) to the mixture and the light emission was recorded every second for 15 min. in a luminometer (Berthold, Germany). The activity was expressed as relative light units (RLU)/µg of total protein.

Quantification of reactive oxygen species (ROS)

The generation of ROS in hSMC was measured by 2,7-dichlorofluorescein (DCF) fluorescence technique [26]. Briefly, cultured cells were scrapped and resuspended in HEPES-buffered saline solution, pH 7.4, containing (in mM): NaCl, 145; KCl, 5; CaCl₂, 1.8; MgCl₂, 1; Na₂HPO₄, 1; glucose, 5; HEPES, 25. After loading with 5 µmol/l DCF for 30 min. at 37°C, the cells were distributed at 10⁴ cells/well into a 96-well microplate reader (Tecan, Austria). DCF fluorescence emission was detected at 535 nm with an excitation wavelength of 485 nm. The ROS production was expressed as relative fluorescence units (RFU)/µg of total protein.

Quantification of the gene expression for LDL receptors, NADPH oxidase subunits and MCP-1

Total RNA was isolated from hSMC using the GenElute Mammalian Total RNA Kit (Sigma). The M-MLV Reverse Transcriptase PCR Kit (Invitrogen, Carlsbad, CA, USA) was used to perform the reverse-transcription of RNA and the amplification of complementary DNA (cDNA). The gene expression for LDL receptor (LDL-R), LDL receptor related protein1 (LRP1), scavenger receptor CD36, NADPH subunits (NOX1, NOX4, p67^{phox}, and p22^{phox}) and monocyte chemoattractant protein-1 (MCP-1) was assessed by Real-time PCR (using an Opticon 2 DNA Engine Real-Time thermocycler -MJ Research, USA) using specific primers (Table 1). GAPDH or 28S rRNA was used as housekeeping gene. The PCR reactions were optimized for each pair of primers in order to obtain a single specific reaction product. Optimized amplification conditions were 0.2 µmol/l of each primer, and 2.5 mmol/l MgCl₂. Quantification of the PCR products was done using the 'Fit Point Method' [27], and expressed as arbitrary units (AU). The efficiency of the amplification reaction of target and internal standard were similar. The cDNA was amplified through 35–40 cycles (30 sec., 94°C; 30 sec., 56°C for CD36, 62°C for LDL-R and 60°C for NOX1, NOX4, p67^{phox}, p22^{phox}, LRP1, RAGE and MCP-1; the SyBr green fluorescent complex continuously read; 1 min., 72°C), followed by a melting curve program (from 55°C to 94°C, with compound read at every 1°C) and finally a cooling step at 4°C.

Quantification of the protein expression by Western blot analysis

Cells were lysed in Radiolmmuno Precipitation Assay (RIPA) buffer containing 50 mMTris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride, pH 7.4, 1% Triton X-100, 1% Na-deoxycholate, 0.1% sodium dodecyl sulphate. The proteins (50 µg) were separated on 8%

SDS-PAGE, transferred to a nitrocellulose membrane and blocked over night with 5% non-fat milk and 0.05% Tween 20 in Tris-buffered saline pH 7.5. The blots were then exposed to the primary antibodies against human CD36 (rabbit polyclonal), RAGE (mouse monoclonal), MCP-1 (rabbit polyclonal) and β -actin (mouse monoclonal), followed by horseradish peroxidase conjugated goat anti-rabbit or anti-mouse IgG; all antibodies were from Abcam, UK, except for β -actin (Santa Cruz, USA) and the ECL reagents (Pierce, Rockford, IL, USA). The relative protein expression was normalized to β -actin after densitometry with TotalLab100 software. For soluble MCP-1, the medium was collected after 24 hrs incubation of hSMC with LDL, concentrated by ultrafiltration and 10 μ l aliquots were applied on 15% SDS-PAGE.

Statistical analysis

Data were expressed as means \pm SD. Statistical evaluation was done by one-way ANOVA test; $P \leq 0.05$ was considered statistically significant. Standard chemicals and reagents were purchased from Sigma (Saint Louis, MO, USA) if not otherwise stated.

Results

Characterization of AGE-LDL

The extent of LDL glycation was estimated using TNBSA, which upon coupling to free, non-glycated amino groups gives the highly chromogenic 2,4,6-trinitrophenyl (TNP) derivatives. Compared to nLDL (0.50 ± 0.015 AU) or oxLDL (0.44 ± 0.055 AU), the assay revealed a 40% reduction of the free amino groups of apoB in AGE-LDL (0.30 ± 0.00 AU, $P < 0.05$) indicative of the irreversible glycation of the protein (Fig. 1A). In addition, the TBA assay indicated the extensive glycation of apoB: a 4-fold increased glycation of AGE-LDL (6.80 ± 2.73 nmoles HMF/mg protein, $P < 0.05$) compared to nLDL (1.81 ± 1.34 nmoles HMF/mg protein) (Fig. 1B). A 2-fold increase of the HMF content in oxLDL (4.68 ± 1.40 nmoles HMF/mg protein) was determined. Measurement of the FFI characteristic fluorescence showed a 10-fold increase in AGE-LDL (1.35 ± 0.01 AU, $P < 0.01$) and oxLDL (2.08 ± 0.12 AU, $P < 0.01$) compared to nLDL (0.17 ± 0.06 AU) (Fig. 1C). Pentosidine content was increased in AGE-LDL (1.64 ± 0.32 AU, $P < 0.05$) and oxLDL (1.52 ± 0.28 AU, $P < 0.05$) compared to nLDL (0.76 ± 0.05 AU) (Fig. 1C).

The mean lipid peroxides level was 5-fold higher in AGE-LDL (1.77 ± 0.5 nmoles MDA/mg protein, $P < 0.05$) and 10-fold higher in oxLDL (4.06 ± 0.02 nmoles MDA/mg protein) than in nLDL (0.35 ± 0.23 nmoles MDA/mg protein).

The Lp charge was determined by agarose gel electrophoresis. Measurement of the Rf showed a 29% increase for AGE-LDL (0.71 ± 0.06 , $P < 0.05$) and a 10% increase for oxLDL (0.61 ± 0.06 , $P < 0.05$) compared with nLDL (0.55 ± 0.08) (Fig. 1D). The increase in the overall negative charge of AGE-LDL was indicative of the loss of positive charges due the glycation of Lys and Arg

residues. All LDLs appeared as a single band indicating the lack of contamination with other proteins.

Regulation of NADPH oxidase activity

To assess further the pro-oxidant effect of AGE-LDL (compared to nLDL) on hSMC, NADPHox activity was determined employing the lucigenin-enhanced chemiluminescence assay. The results revealed that the NADPHox activity was significantly enhanced in hSMC by AGE-LDL ($35,530 \pm 381$ RLU/ μ g protein, $P < 0.05$) and oxLDL ($34,169 \pm 467$ RLU/ μ g protein, $P < 0.05$); in contrast, the enzyme activity was comparable in control cells ($26,970 \pm 280$ RLU/ μ g protein) and in cells incubated with nLDL ($29,431 \pm 400$ RLU/ μ g protein) (Fig. 2A).

Modulation of intracellular ROS production

The oxidative effect of native and AGE-LDL was analysed in quiescent hSMC incubated for 24 hrs with 100 μ g LDLs protein/ml employing DCFA cellular loading. The results showed that the intracellular ROS production increased significantly in cells exposed to AGE-LDL (2198 ± 30 RFU/ μ g protein, $P < 0.05$) as compared to control cells (1600 ± 22 RFU/ μ g protein) or cells incubated with nLDL (1715 ± 28 RFU/ μ g protein) (Fig. 2B).

Regulation of gene and protein expression for NOX1, NOX4, p22^{phox} and p67^{phox}

To identify which of the NADPHox subunits is implicated in the enhanced enzyme activity, Real-Time PCR was employed to assess the gene expression of NADPHox subunits upon exposure of hSMC to nLDL, AGE-LDL or oxLDL. In comparison with nLDL, AGE-LDL induced a significantly increased level of mRNA for NOX1 (~80%, $P < 0.05$), NOX4 (~60%, $P < 0.05$) and p67^{phox} (~60%, $P < 0.05$) (Fig. 3A–D). Stimulation of hSMC with oxLDL resulted in a higher increase of NOX1, NOX4, p22^{phox} and p67^{phox} mRNA over the AGE-LDL level.

Western blotting employing anti-NOX1 and anti-NOX4 antibodies revealed a significant increase in the protein expression of NOX1 for AGE-LDL (0.54 ± 0.03 , $P < 0.05$) exposed hSMC relative to control cells (0.33 ± 0.04). The data are in good agreement with the increase of NOX1 gene expression. The protein expression of NOX4 was significantly augmented in hSMC incubated with oxLDL (0.69 ± 0.12 , $P < 0.05$), while AGE-LDL induced a smaller increase (0.48 ± 0.13) compared to control cells (0.34 ± 0.03).

AGE-LDL induces lipid loading of hSMC

To determine the lipid accumulation in hSMC following exposure to AGE-LDL, nLDL or oxLDL, the cells were stained with lipid specific fluorochrome, Nile Red. Fluorescence microscopy

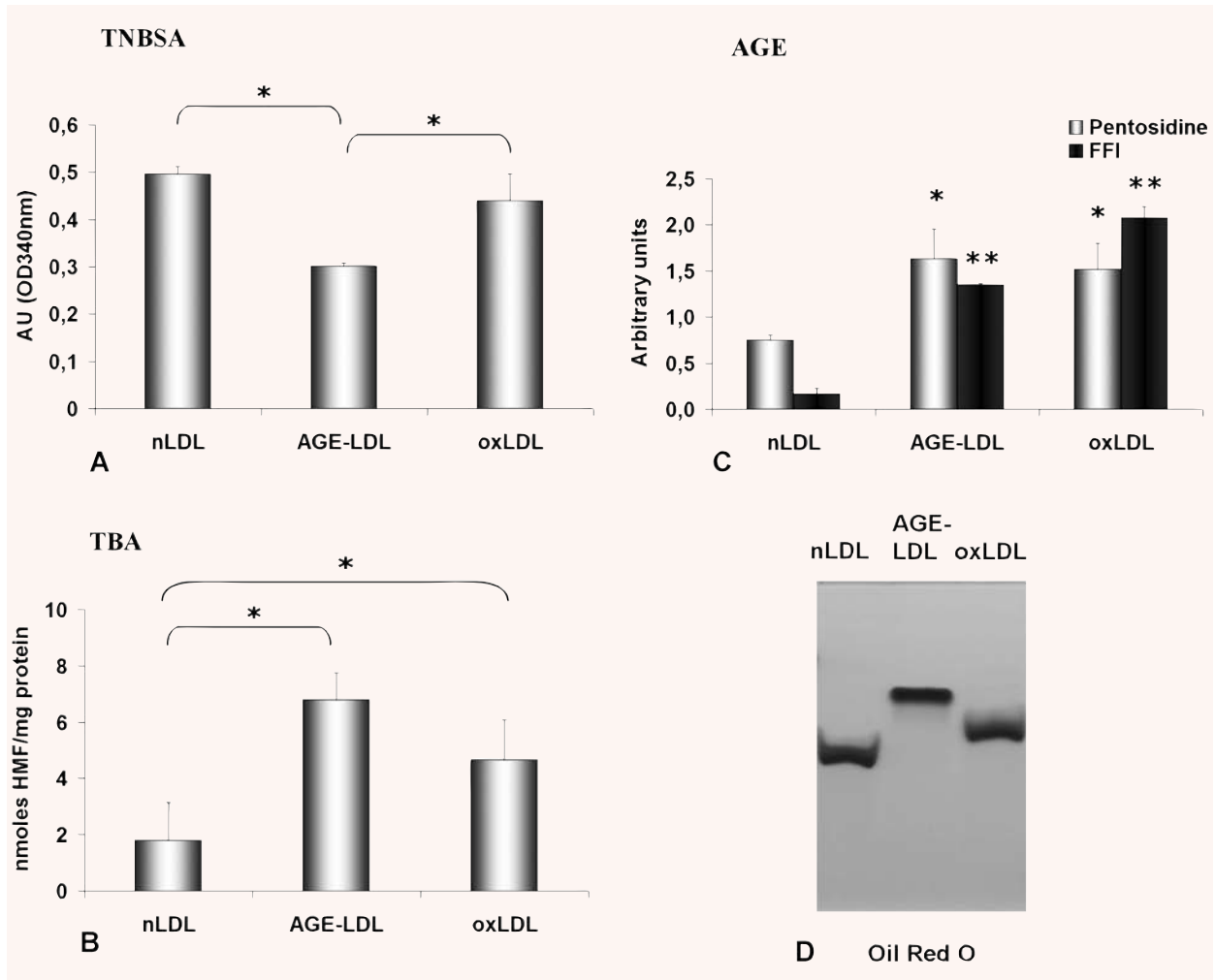


Fig. 1 Characterization of AGE-LDL. Comparative evaluation of native LDL (nLDL), copper-oxidized LDL (oxLDL) and AGE-LDL (obtained by incubation of nLDL with 0.2M D(+)-glucose for 4 weeks). The glycated epitopes were measured by TNBSA (A) and TBA (B) assays, pentosidine and FFI levels (C), $*P < 0.05$, $**P < 0.01$, $n = 4$, and the electronegative charge in 0.6% agarose gel electrophoresis (Oil Red O staining) (D).

revealed a considerably increased number of fluorescent lipid droplets in cells incubated with AGE-LDL compared to control cells or cells incubated with nLDL (Fig. 4 A–C) or oxLDL (Fig. 4D). Enzymatic measurement of the intracellular cholesteryl esters (CE) accumulation in cells incubated for 24 hrs with 25, 50, 100 and 150 $\mu\text{g/ml}$ AGE-LDL indicated that the maximum level of cholesterol was at 100 $\mu\text{g/ml}$ glycated LDL in the medium. Time-course determination of the intracellular CE accumulation at 12, 24, 36 and 48 hrs indicated that the maximum level of cholesterol was at 24 hrs incubation, and the CE/TC ratio increased in cells exposed to 100 $\mu\text{g/ml}$ AGE-LDL (30.08 ± 5.54 , $P < 0.05$) compared to nLDL (16.68 ± 3.67) or oxLDL (20.06 ± 2.54). These data corroborate well with those obtained by lipid staining.

LDL receptors, CD36 and RAGE gene expression

The gene expression of LDL-R, LRP1, CD36 and RAGE in hSMC incubated with AGE-LDL or nLDL for 24 hrs was quantified by quantitative PCR.

The gene expression of LDL-R was significantly inhibited by exposure of cells both to nLDL (18 ± 2 , $P < 0.001$) and to AGE-LDL (28 ± 7 , $P < 0.01$), compared to control hSMC (considered 100) (Fig. 5A).

The LRP1 gene expression increased 2.6-fold (172 ± 43 , $P < 0.05$) in AGE-LDL-exposed cells compared to cells incubated with nLDL (65 ± 33) (Fig. 5B).

AGE-LDL determined an increase of the gene expression for scavenger receptor CD36 with 35% (169 ± 11 , $P < 0.05$)

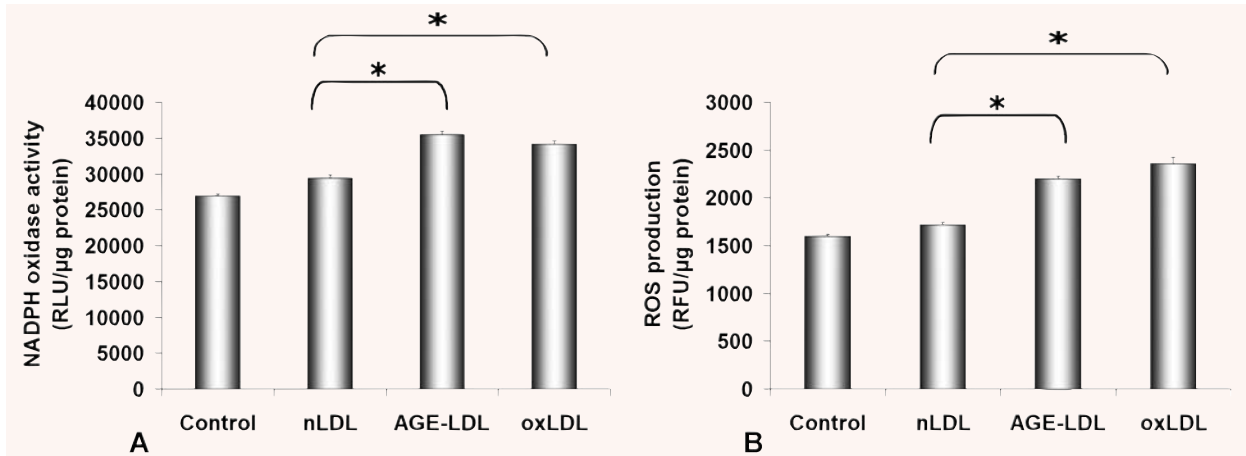


Fig. 2 Oxidative stress induced by AGE-LDL incubation with hSMC. NADPH oxidase activity (A) was measured by lucigenin-enhanced chemiluminescence (A), and reactive oxygen species (ROS) production by dichlorofluoresceine assays (B). 100 μg protein/ml AGE-LDL, oxidized LDL (oxLDL) or native LDL (nLDL) were incubated 24 hrs with hSMC. Control, cells not exposed to LDLs, **P* < 0.05, *n* = 3.

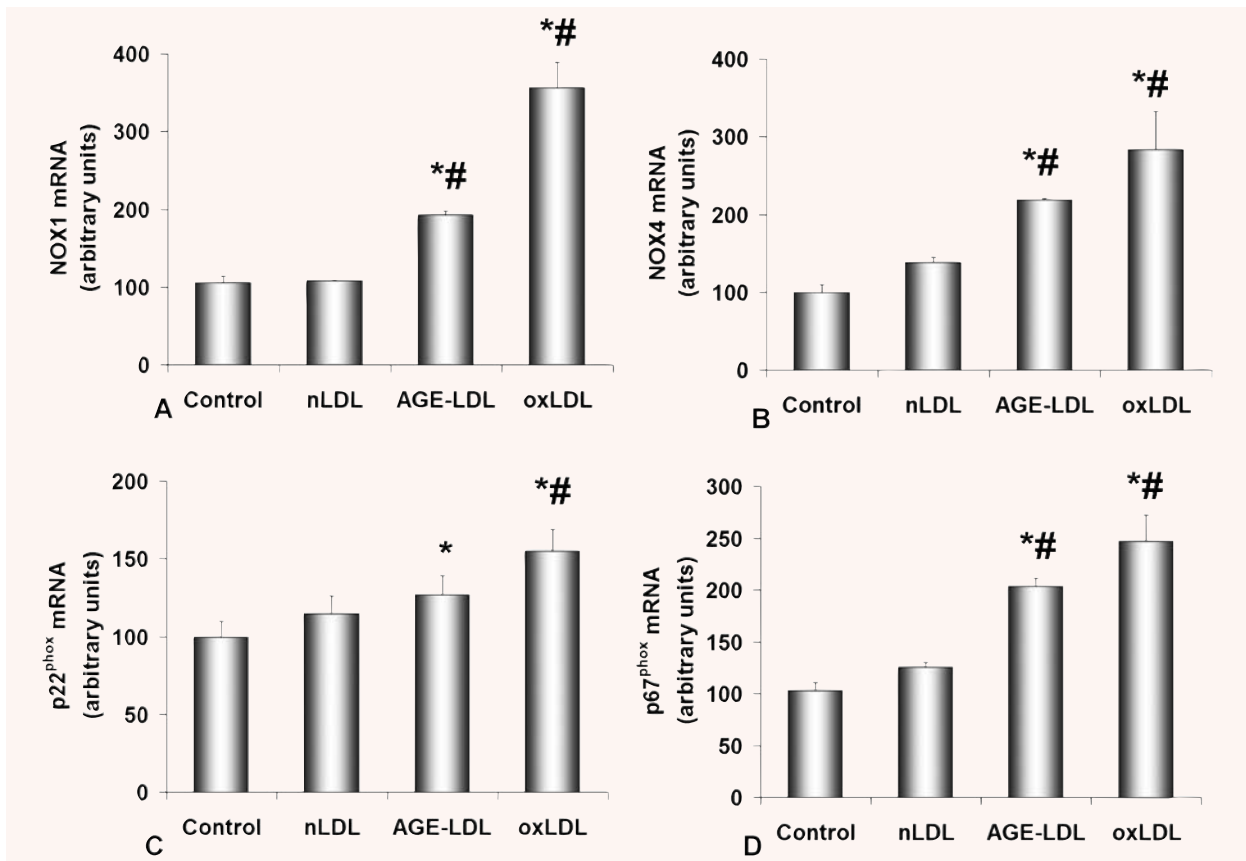


Fig. 3 AGE-LDL up-regulates NADPH oxidase subunits expression in hSMC. Expression of mRNA obtained by Real-time PCR for NOX1 (A), NOX4 (B), p22^{phox} (C) and p67^{phox} (D) relative to control cells, not exposed to LDL. GAPDH was the reference gene, AGE-LDL, oxidized LDL (oxLDL) and native LDL (nLDL) concentration was 100 μg/ml and incubation time 24 hrs. Control, cells not exposed to LDL, **P* < 0.05 versus control cells, #*P* < 0.05 versus nLDL, *n* = 3.

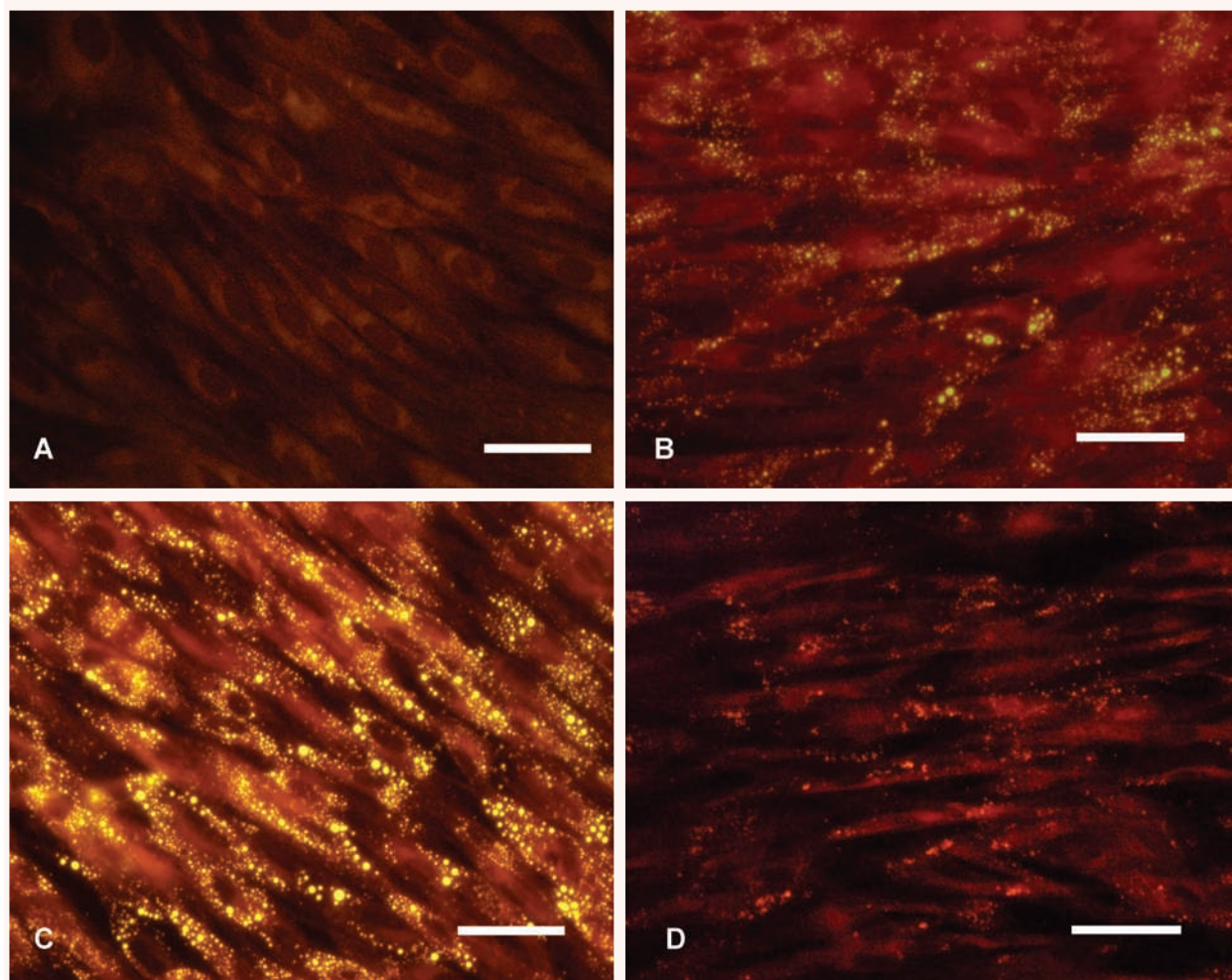


Fig. 4 AGE-LDL induces lipid accumulation in hSMC. The cells were incubated with 100 $\mu\text{g/ml}$ AGE-LDL, oxidized LDL (oxLDL) or native LDL (nLDL) for 24 hrs, fixed in 2% formaldehyde and stained with Nile Red (yellow fluorescence). **(A)** Control, cells not exposed to LDL, **(B)** cells exposed to nLDL, **(C)** AGE-LDL and **(D)** oxLDL; bar = 25 μm , $n = 3$.

above the values obtained for nLDL-exposed cells (126 ± 14) (Fig. 5C).

Results of the quantification of RAGE mRNA indicated a 10% increase of the gene expression in both LDLs exposed cells compared to control ones, but this was not statistically significant (Fig. 5D).

LRP1, CD36 and RAGE protein expression

Incubation of hSMC with AGE-LDL (24 hrs) induced a 32% increase of LRP1 protein expression (0.90 ± 0.13 , $P < 0.05$) relative to nLDL (0.68 ± 0.08), whereas oxLDL caused a 57% (1.07 ± 0.04 , $P < 0.05$) increase (Fig. 6A); the data are in good accordance with the raise of the gene expression of LRP1.

In similar experimental conditions, the results showed that AGE-

LDL induced a significantly increased protein expression of CD36 (0.96 ± 0.02 , $P < 0.05$) compared to nLDL (0.40 ± 0.18), as illustrated in the blots and corresponding histograms (Fig. 6B). The augmentation of the CD36 protein expression was in good agreement with the measured increase of the CD36 mRNA. Under the same conditions, AGE-LDL induced an increased expression of RAGE protein (0.14 ± 0.03 , $P < 0.001$) compared to nLDL (Fig. 6C).

MCP-1 gene and protein expression

The results obtained by quantitative PCR showed that upon incubation of hSMC with AGE-LDL, the gene expression of MCP-1 increased 1.6-fold (256 ± 35 , $P < 0.05$) above control

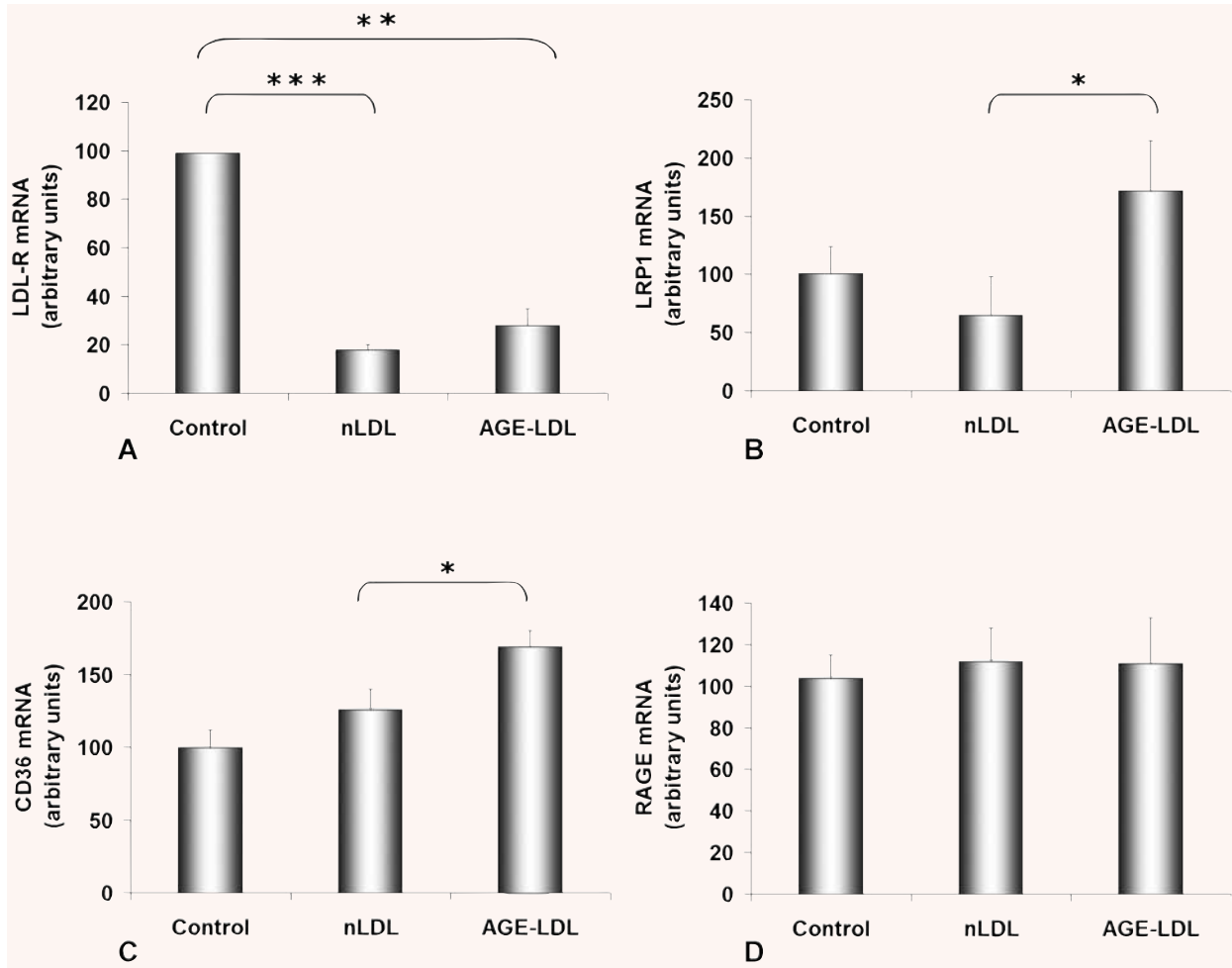


Fig. 5 AGE-LDL modulates the gene expression of LDL receptors in hSMC. Cells were incubated for 24 hrs with 100 $\mu\text{g/ml}$ native LDL (nLDL) or AGE-LDL and then subjected to quantitative PCR for LDL receptor (LDL-R, **A**), LDL receptor-related protein 1 (LRP1, **B**), scavenger receptor CD36 (**C**) and AGE-receptor (RAGE, **D**); 28S rRNA was the reference gene. Control, cells not exposed to LDL, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, $n = 3$.

values, whereas the effect of nLDL was significantly lower (158 ± 5) (Fig. 7A).

A significant increase of MCP-1 protein released in the culture medium was detected after hSMC were incubated with AGE-LDL (215 ± 31 , $P < 0.05$) compared to nLDL (70 ± 5) (Fig. 7B). In contrast, analysis of the protein expression of MCP-1 in cell lysates revealed no specific band on the Western blot.

The effect of AGE-LDL on hSMC viability, metabolic competence and proliferation

Viability of hSMC after exposure (24 hrs) to AGE-LDL, nLDL or oxLDL evaluated by the MTT test showed that the cells were not

affected by the exposure to either of the LDLs, as compared to the viability of control cells (data not shown).

The energy metabolism test showed no significant change in ATP/DNA content when hSMC were incubated with AGE-LDL (1729 ± 151 RFU/ μg DNA) relative to nLDL (1863 ± 84 RFU/ μg DNA). The ATP content of cells incubated with oxLDL significantly decreased (1102 ± 257 RFU/ μg DNA) as compared to nLDL. Results on the metabolic competence of treated cells indicated no changes in glucose uptake when cells were incubated with AGE-LDL (29.26 ± 1.47 mg glucose consumed/ μg DNA) or oxLDL (28.85 ± 0.63 mg glucose consumed/ μg DNA) compared to control cells (29.36 ± 0.84 mg glucose consumed/ μg DNA) or cells incubated with nLDL (28.94 ± 0.84 mg glucose consumed/ μg DNA).

Determination of the cellular DNA content of hSMC incubated for 24 hrs with 25, 50, 100 and 150 $\mu\text{g/ml}$ AGE-LDL indicated that the

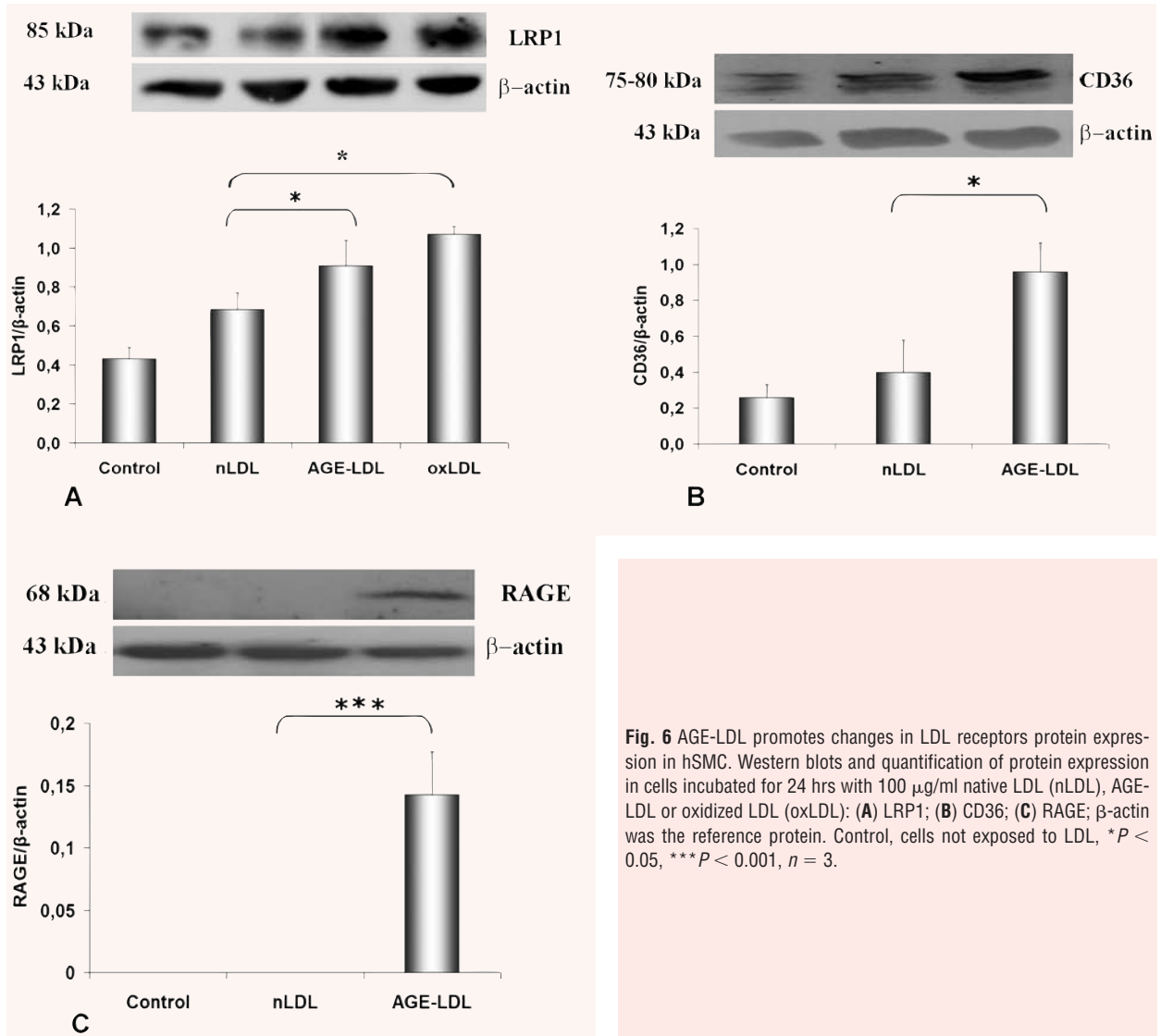


Fig. 6 AGE-LDL promotes changes in LDL receptors protein expression in hSMC. Western blots and quantification of protein expression in cells incubated for 24 hrs with 100 μ g/ml native LDL (nLDL), AGE-LDL or oxidized LDL (oxLDL): (A) LRP1; (B) CD36; (C) RAGE; β -actin was the reference protein. Control, cells not exposed to LDL, * P < 0.05, *** P < 0.001, n = 3.

maximum proliferation rate was at 100 μ g/ml glycosylated LDL in the medium. Time-course determination of the cellular DNA content of hSMC incubated with 100 μ g/ml AGE-LDL for 12, 24, 36 and 48 hrs indicated a maximum rate of proliferation at 24 hrs. Results showed that AGE-LDL induced a 30% increase ($49.57 \pm 4.42 \mu$ g DNA, P < 0.01) of the cells proliferation rate at 24 hrs, while oxLDL induced a 48% increase ($56.87 \pm 3.84 \mu$ g DNA, P < 0.001) relative to the cells exposed to nLDL ($38.45 \pm 1.44 \mu$ g DNA) (Fig. 8).

Discussion

In diabetes, dysfunction of the cells of the vessel wall may be in part the result of their interaction with glycosylated proteins, such as

AGE-LDL, present concurrently in the plasma and in the intima. AGE-LDL is detected both in hyperlipidaemic and hyperglycaemic patients' plasma [28, 29]. The effect of AGE-LDL on vascular SMC is not known. To detect the alterations generated by AGE-LDL on hSMC, we looked for the NADPHox activity and the intracellular ROS production, the accumulation of lipid droplets and the implication of specific receptors (LRP1, CD36, RAGE), the induction of MCP-1 and the modulation of the proliferation rate.

We chose to incubate the cells with 10 mg AGE-LDL protein/dl culture medium that is in the physiological range (9.3 mg/dl glycosylated LDL apoB in the serum of patients with diabetes versus 4.8 mg/dl in controls) [28]. Interestingly, Akanji *et al.* [29] reported that serum-glycosylated LDL level is increased in patients with hyperlipidaemia without diabetes (6.12 mg/dl), and further increased in patients with both hyperlipidaemia and diabetes

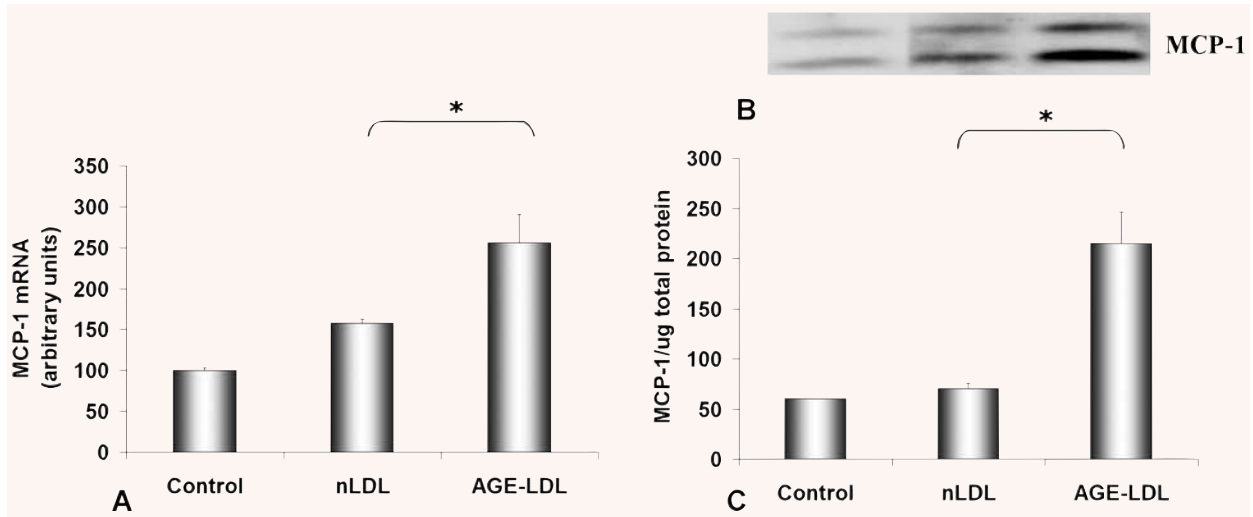


Fig. 7 AGE-LDL stimulates MCP-1 gene and protein expression in hSMC. Cells were incubated for 24 hrs with 100 μ g/ml native LDL (nLDL) or AGE-LDL and then subjected to quantification of MCP-1 gene expression relative to 28S rRNA (A), and Western blot of soluble MCP-1 protein relative to the total protein in the medium (B). Control, cells not exposed to LDL, * $P < 0.05$, $n = 3$.

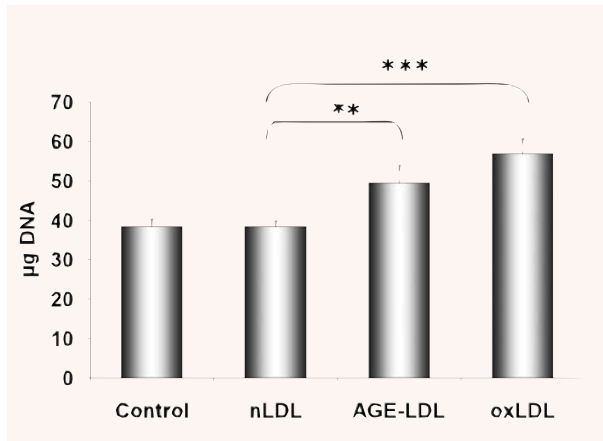


Fig. 8 Increased proliferation rate of hSMC induced by AGE-LDL. Cells were incubated for 24 hrs with 100 μ g protein/ml AGE-LDL, oxidized LDL (oxLDL) or native LDL (nLDL). Cell proliferation was assessed by Hoechst 33258 fluorimetric assay. Control, cells not exposed to LDL, ** $P < 0.01$, *** $P < 0.001$, $n = 3$.

(7.82 mg/dl) compared with 2.74 mg/dl in healthy subjects. It has been suggested that glycated LDL apoB could be utilized as an index of medium-term glycaemic control as it correlates with glucose, fructosamine and glycated haemoglobin (HbA1c) in patients with diabetes [30].

It was reported that in the vasculature, the up-regulation of NADPHox activity is often associated with the progression of vascular disorders related to hypertension, diabetes or obesity, and is

correlated with increase in NOX1 mRNA level. Unlike NOX1, which requires regulatory subunits for its activity, NOX4 produces ROS constitutively and changes in its gene expression affect directly the NOX4 activity [31].

In our experiments, the pro-oxidant potential of AGE-LDL on hSMC was substantiated by the pronounced increase of the gene expression of NADPHox subunits (NOX1, NOX4, p22phox, p67phox) compared to cells exposed to nLDL, and this correlated well with an enhanced superoxide production driven by the activated NADPHox. The demonstrated AGE-LDL stimulated protein expression of RAGE may be part of the molecular mechanism accountable for the increased ROS production in hSMC by activation of protein kinase C and the increased activity of NADPHox, considered to be key events by which AGEs trigger ROS. Our data indicate that AGE-LDL induces up-regulation of NOX1 and NOX4; these may have an important role in promoting vascular SMC growth, proliferation and differentiation [32]. The p22phox subunit is essential for the NADPHox activity and it was demonstrated [33] that p22phox is more abundant in advanced atherosclerosis plaques than in non-atherosclerotic arteries, suggesting a correlation between p22phox expression, superoxide production and the severity of atherosclerotic lesions. In previous studies, we demonstrated that NF-kB and AP-1 regulate p22phox gene expression in hSMC [34, 35]. The p67phox is regulated also by the same transcription factors [36, 37]. We can suggest that the regulation of p22phox and p67phox expression by AGE-LDL in hSMC may be mediated through an NF-kB or AP-1-dependent mechanism.

AGE-LDL induced lipid loading and cholesteryl esters accumulation in hSMC, having an optimum dose at 100 μ g/ml AGE-LDL and 24 hrs incubation, in good agreement with Tirziu *et al.* and Llorente-Cortes *et al.* [21, 38]. To uncover the possible mechanisms involved

in this process, we searched for the expression of native and modified LDL receptors. We report here that AGE-LDL induces concurrently a decrease in the gene expression for the LDL receptor, and a significant increase in LRP1 expression. To the best of our knowledge, this is the first report indicating the significant augmentation of LRP1 gene and protein expression induced by AGE-LDL in vascular hSMC. The decreased recognition of glycated LDL by the LDL-R correlates directly with the degree of apoB glycation, and may explain the increased intimal retention of glycated Lp [39], formation of AGE products and the activation of alternative uptake mechanisms (non-LDL-R pathways) in vascular cells of diabetic patients [40]. It was reported that *in vitro* aggregated LDL stimulates LRP1 expression and is internalized by the LRP1 pathway [38].

In diabetic patients, chronic hyperglycaemia enhances glucose-induced LDL glycooxidation, both in circulation and in the vessel wall. We have demonstrated the increased expression of CD36 in hSMC exposed to AGE-LDL. This result adds to the previous reports that AGE-proteins are recognized by the CD36 receptor [41] and its protein expression is higher in type 2 diabetic rat aortas than in controls [42].

AGE-LDL induced increased protein expression of RAGE in hSMC, without affecting the RAGE gene expression. It was reported that RAGE mRNA levels do not appear to correlate closely with levels of RAGE antigen, suggesting that there are likely to be multiple factors regulating translation, post-translational processing, and degradation of RAGE [43].

Taken together our data, we can predict that the AGE-LDL-induced augmented expression of LRP1, CD36, and RAGE in hSMC contributes to the increased uptake of modified lipoproteins in the neointima and the progression of diabetic atherosclerosis.

Data from this study show that AGE-LDL generates an increase of MCP-1 gene expression in hSMC and of MCP-1 protein released from the cells in the culture medium. It was reported that AGE-LDL induces expression of MCP-1 receptor (CCR2) in monocytes and thus can stimulate the chemotactic response elicited by MCP-1 [44]. Thus, we can suggest that AGE-LDL-induced expression of MCP1 in hSMC and that of the matching receptor on monocytes/macrophages contributes significantly to the infiltration of plasma

monocytes into the neointima and to the interaction between SMC and macrophages within the atherosclerotic plaque.

In our experiments, the energy metabolism of hSMC, expressed as intracellular ATP/ μ g DNA content, was not affected by exposure to AGE-LDL relative to nLDL, while in cells incubated with oxLDL the level was decreased, in good agreement with previous data [45]. The metabolic competence of hSMC showed no change in cellular glucose/ μ g DNA consumption when cells were incubated with AGE-LDL or oxLDL. A similar report on the effect of AGE-proteins on hSMC glucose metabolism was presented by Ballinger [46] on AGE-BSA-treated macrophages.

Incubation of AGE-LDL with hSMC induced a significant increase in the proliferation rate (30%) compared to cells exposed to nLDL. This effect can be correlated with the AGE-LDL-induced NOX4 up-regulation, which is known to have an important role in promoting vascular SMC proliferation. This result corroborates and extends previous data showing that glycated LDL directly stimulates SMC proliferation [47] and AGEs induce proliferation of cultured rat vascular SMC [48]. We suggest that AGE-LDL, by promoting the proliferation of SMC in the neointima, contributes to the accelerated atherosclerotic plaque formation in diabetic patients.

In summary, the results of this study indicate that AGE-LDL activates hSMC by up-regulation of receptors such as LRP1, CD36, RAGE and the subsequent lipid accumulation, determines a pro-oxidant state by activation of NADPHox, and a pro-inflammatory state expressed by the induction of MCP-1. Understanding the outcome of the association of irreversible glycation and oxidation of LDL, two critical processes occurring in diabetes, will guide the design of novel or complementary therapeutic strategies to prevent or treat this disease.

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