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Electronegative LDL induces MMP-9 and TIMP-1 release in monocytes through CD14 activation: Inhibitory effect of glycosaminoglycan sulodexide

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ABSTRACT

Objective: Electronegative LDL (LDL(−)) is involved in atherosclerosis through the activation of the TLR4/CD14 inflammatory pathway in monocytes. Matrix metalloproteinases (MMP) and their inhibitors (tissue inhibitors of metalloproteinase [TIMP]) are also crucially involved in atherosclerosis, but their modulation by LDL(−) has never been investigated. The aim of this study was to examine the ability of LDL(−) to release MMPs and TIMPs in human monocytes and to determine whether sulodexide (SDX), a glycosaminoglycan-based drug, was able to affect their secretion.

Approach and results: Native LDL (LDL(+)) and LDL(−) separated by anion-exchange chromatography were added to THP1-CD14 monocytes in the presence or absence of SDX for 24 h. A panel of 9 MMPs and 4 TIMPs was analyzed in cell supernatants with multiplex immunoassays. The gelatinolytic activity of MMP-9 was assessed by gelatin zymography. LDL(−) stimulated the release of MMP-9 (13-fold) and TIMP-1 (4-fold) in THP1-CD14 monocytes, as well as the gelatinolytic activity of MMP-9. Co-incubation of monocytes with LDL(−) and SDX for 24 h significantly reduced both the release of MMP-9 and TIMP-1 and gelatinase activity. In THP1 cells not expressing CD14, no effect of LDL(−) on MMP-9 or TIMP-1 release was observed. The uptake of DiI-labeled LDL(−) was higher than that of DiI-LDL(+) in THP1-CD14 but not in THP1 cells. This increase was inhibited by SDX. Experiments in microtiter wells coated with SDX demonstrated a specific interaction of LDL(−) with SDX.

Conclusions: LDL(−) induced the release of MMP-9 and TIMP-1 in monocytes through CD14. SDX affects the ability of LDL(−) to promote TIMP-1 and MMP-9 release by its interaction with LDL(−).

1. Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases crucially involved in the remodeling of the vascular extracellular matrix (ECM) and in the unmasking of several ECM-bound cytokines and growth factors [1,2]. MMP activities are thoroughly orchestrated by control points at transcriptional, post-transcriptional, and post-translational levels, these latter including regulated activation of zymogen pro-forms, as well as inhibition through interaction with endogenous tissue inhibitors of metalloproteinases (TIMP-1, -2, -3, and -4) [1]. MMPs play key roles in the development and regulation of the physiological processes of the cardiovascular system and in the aberrant remodeling of the vasculature and atherosclerotic plaque formation and instability [3].

Besides MMP imbalance, one of the major initiators involved in the development of atherosclerotic plaque is the chemical modification of

Abbreviations: ECM, extracellular matrix; GAG, glycosaminoglycan; IL, Interleukin; LDL, low density lipoprotein; LPS, lipopolysaccharide; LSU, lipasemic unit; MMP, matrix metalloproteinase; SDX, sulodexide; TIMP, tissue inhibitor of metalloproteinase; TLR, toll-like receptor

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lipids. In particular, low density lipoprotein (LDL) modification has been established as an important risk factor in the activation of many atherogenic pathways [4]. In this respect, electronegative LDL (LDL (−)) is a minor modified LDL subfraction present in blood circulation, mainly in patients with high cardiovascular risk factors [5]. LDL(−) differs from electropositive LDL (LDL(+)) in size, density, lipid content, protein composition, and apo-B100 conformation [6,7].

An expanding body of evidence supports the pro-inflammatory role of LDL(−) [5,8–12]. First identified in endothelial cells [13] and later in monocytes [9–12,14,15], a number of studies have described the ability of LDL(−) to induce cytokine release in monocytes [10,11,14] by activating the signaling cascade downstream of CD14/toll-like receptor 4 (TLR4) [9,12,15]. In this respect, it has been reported that MMPs are induced by CD14-TLR activation in monocytic cells [16]; however, although the ability of LDL(−) to stimulate MMP release has been previously investigated in in vitro models of endothelial cells [17], no data are available regarding the modulation by LDL(−) of proteolytic pathways in monocytic cells. The first aim of this study was to evaluate the effects of LDL(−)-treatment on THP1 cells over expressing CD14 in promoting the release of MMPs and their inhibitors.

Another particular feature of LDL(−) is an increased affinity for glycosaminoglycans (GAGs) [6,18,19]. Some GAGs are widely used in vascular medicine for their endothelial protective and anti-inflammatory properties. Sulodexide® (Vessel™, Alfa-Sigma, Italy) is a highly purified mixture of two GAGs, composed of 80% fast-moving heparin (FMH), which has affinity with antitrombin III (ATIII), and 20% dermatan sulfate, which has affinity with the heparin cofactor II (HCII) [20]. Both of these components confer anti-thrombotic and anti-coagulant effects. Moreover, sulodexide (SDX) has endothelial protective and anti-inflammatory properties due to its interaction with the glycocalyx layer of blood vessels and its ability to modulate inflammatory pathways in monocytes [21–23]. Notably, one of the first characterized properties of SDX was its lipoprotein lipase releasing ability [24,25], which endows SDX with cholesterol-lowering properties and the ability to modify lipoprotein catabolism, strengthening the basis for conducting studies on the use of SDX as a potential anti-atherosclerotic agent. Because it is known to increase the affinity of LDL(−) for GAGs [6,18,19], the authors of this study assessed how SDX affects the release of MMPs and TIMPs in monocytes stimulated with LDL(−).

2. Materials and methods

2.1. Materials

Pure grade chemicals and reagents for the MMP multiplex immunoassays were obtained from Bio-Rad (Milan, Italy). Commercial SDX was provided from Alfa-Sigma (Italy). All the reagents for the zymography assays were obtained from Bio-Rad, excluding 90 Bloom gelatin type A, derived from porcine skin, which was obtained from Sigma-Merck (Darmstadt, Germany). An Amplex Red cholesterol kit was obtained from Sigma. THP1 and **THP1-XBlue™-MD2-CD14 cells** were obtained from Invivogen (Toulouse, France).

2.2. Lipoprotein isolation and separation of LDL subfractions

Plasma samples from healthy normolipemic subjects (total cholesterol < 5.2 mM, triglyceride < 1 mM) were obtained in EDTA-containing Vacutainer tubes. Total LDL (1.019–1.050 g/mL) was isolated from pooled plasma by sequential flotation ultracentrifugation at 4 °C in the presence of 1 mM EDTA and 2 μM BHT, and LDL was dialyzed against buffer A (Tris 10 mM, EDTA 1 mM, pH 7.4). Native LDL (LDL(+)) and LDL(−) were separated by preparative anion-exchange chromatography in an ÄKTA-FPLC system (Amersham Pharmacia, Uppsala, Sweden) and characterized as described [26]. The LDL(−) proportion ranged from 4 to 6% of total LDL in all LDL preparations. The physicochemical characteristics and composition of both LDL subfractions were similar to those previously reported [26]. Briefly, LDL(−) contained more triglycerides and non-esterified fatty acids, presented smaller particle size, and demonstrated higher aggregation than LDL(+). No difference in the oxidative level between LDL(+) and LDL(−) was observed (data not shown).

2.3. Cell culture and incubation

THP1-XBlue™-MD2-CD14 cells (Invivogen) (THP1-CD14) are derived from THP1 human monocytic cells over expressing MD2 and CD14 to increase the response to CD14-TLR ligands, such as LDL(−). Cells were grown as previously described [9]. THP1 cells were grown under the same conditions as THP1-CD14 cells, except for the absence of selective antibiotics in RPMI growth medium. LDL(+) and LDL(−) were dialyzed against serum-free RPMI media, filtered in sterile conditions, and added (70 mg apoB/L) to THP1-CD14 cells (400,000 cells/mL) in serum-free media. In order to evaluate the effects of SDX on LDL (−)-induced response, monocytes were co-incubated with SDX (0.12 LSU/mL) and LDL(−) simultaneously. After 24 h of incubation, cell supernatants were collected and stored at −80 °C until analysis.

2.4. Magnetic multiplex immunoassays of MMP-9 and TIMP-1

Levels of MMP and TIMP released in THP1 serum-free culture media were determined with the commercially available Pro™ Human MMP 9-plex Assay and the Pro™ Human TIMP 4-plex Assay. MMP-1, -2, -3, -7, -8, -9, -10, -12, and -13 and TIMP-1, -2, -3, and -4 were analyzed. Multiplex suspension immunomagnetic assays are based on the use of fluorescently dyed magnetic beads covalently conjugated with monoclonal antibodies specific to the target proteins, and these assays were performed according to the manufacturer's instructions (Bio-Plex, Bio-Rad Labs, Hercules, CA, USA). Levels of all molecules were determined using a Bio-Plex 200 array reader based on Luminex X-Map Technology (Bio-Rad Labs, Hercules, CA, USA), which detects and quantifies multiple targets in a 96-well plate with a single small fluid volume (50 μL). Data were collected and analyzed using a Bio-Plex 200 instrument equipped with BioManager analysis software (Bio-Plex Manager Software v.6.1). The protein concentrations (expressed as pg/mL) were calculated with a standard curve. According to the manufacturer's data, the lowest detection limits were 1.0 and 1.6 pg/mL for MMPs and TIMPs, respectively.

2.5. Zymographic analyses

Aliquots of all serum-free media (obtained from a cell density of 400,000 cells/mL) were analyzed by gelatin zymography carried out on 6.5% polyacrylamide gels copolymerized with 3 g/L 90 Bloom Type A gelatin from porcine skin (Sigma). Native samples were loaded with the addition of zymogram sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 4% SDS, 0.01% bromophenol blue) [27]. SDS-PAGE gels were run using a Bio-Rad Mini-Protean Tetra Cell apparatus (Bio-Rad, Hercules, CA) in SDS running buffer (25 mM Tris, 192 mM glycine, and 0.1% w/v SDS) at a constant voltage of 105 V. After electrophoresis, gels were incubated for 40 min at room temperature on a rotary shaker in Triton X-100 2.5% to remove SDS. The gels were washed with distilled water and incubated for 24 h in an enzyme incubation buffer (containing 50 mM Tris, 5 mM $CaCl_2$, 100 mM NaCl, 1 mM $ZnCl_2$, 0.3 mM NaN_3, 0.2 g/L of Brij®-35, and 2.5% v/v of Triton X-100, pH 7.6) at 37 °C. Staining was performed using Coomassie Brilliant Blue R-250 (0.2% w/v Coomassie in 50% v/v methanol and 20% v/v acetic acid). Gels were destained with destaining solution (50% v/v methanol and 20% v/v acetic acid) until clear gelatinolytic bands appeared against the uniform dark-blue background of undigested protein substrate. Gelatinase calibrators (as molecular weight standards) were prepared by diluting 1:15 v:v healthy capillary blood with a non-reducing Laemmli sample buffer. It is important to specify that whole

capillary blood, used as calibrator, presents only the zymogens of gelatinases: pro-MMP-2 at 72 kDa, pro-MMP-9 at 92 kDa, and pro-MMP-9 complexes at 130 kDa (MMP-9/NGAL) and 225 kDa (MMP-9 multimeric form), as previously recognized by monoclonal anti-MMP-2 and anti-MMP-9 antibodies and characterized as latent pro-enzymes, activated by *p*-aminophenylmercuric acetate (APMA) and inhibited by both calcium and zinc chelators (EDTA and o-phenanthroline, respectively) [28,29]. Zymographic bands were densitometrically measured with the image analyzer LabImage 1D (Kapelan, Leipzig, Germany).

2.6. Uptake of DiI-labeled LDL(−) by THP1 and THP1-CD14 cells

LDL subfractions were labeled with the fluorescent probe 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes, Eugene, OR) [30]. The specific activity of DiI-LDL (+) and DiI-LDL(−) was determined, and binding experiments were performed essentially as previously reported [30]. Cells (400,000 cells/mL) were incubated with DiI-LDLs (50 mg apoB/L) in serum-free media for 20 h at 37 °C in the absence or presence of SDX (0.12 LSU/mL). After incubation, cells were washed with phosphate buffered saline (PBS) and lipid content was extracted using isopropanol. Fluorescence was measured at an excitation wavelength of 528 nm and an emission wavelength of 578 nm. For fluorescence microscopy analysis, cells were washed with PBS and seeded in a 35-mm μ-dish in an Inverted Zeiss Axiovert 200 M motorized microscope (Carl Zeiss Microscopy, Jena, Germany) with a CCD Photometrics CoolSNAP cf. camera (Metamorph version 5.0r1 software).

2.7. Binding of LDL(−) to SDX

Polystyrene 96-well plates were coated and stored at 4 °C overnight with 50 mU/well of SDX. Free binding sites in SDX-coated wells were then blocked with 3% BSA and 1% fat-free milk powder in PBS for 1 h at 37 °C. For the measurement of non-specific binding, 96-well plates were coated with 5% fat-free BSA in PBS. Increasing concentrations of LDL (50–200 nmol cholesterol) were added for 3 h and 24 h at 37 °C and 5-fold washed with PBS. The amount of bound LDL was measured using an Amplex Red cholesterol kit (Sigma), according to a previous report [19].

2.8. Statistics

Each variable is expressed as mean ± standard deviation (SD) unless otherwise specified. The differences were compared by non-parametric Mann-Whitney or Wilcoxon tests according to variable characteristics. All statistical tests were two-tailed, and significance was set at $p < 0.05$. Data and graphs were analyzed with Prism software for Windows-7, version 5.0 (Graph-Pad, San Diego, CA, USA).

3. Results

3.1. Effects of LDL(−) on MMP and TIMP release by THP1-CD14 cells

MMP multiplex immunoassays have been used to study the potentially different levels of MMP and TIMP secretion promoted by LDL(+) versus LDL(−), focusing particular attention on MMP-9 and its inhibitor TIMP-1, previously described as crucial players in atherosclerotic plaque formation and instability [2,3,31]. In general, the effect of LDLs on MMP release was modest, with LDL(+) showing no effect (MMP-1, -2, -7, -10, -12, -13) or slight decreases (MMP-3, -8). LDL(−) moderately increased the release of MMP-1 (1.7-fold), MMP-12 (2.8-fold), and MMP-13 (1.4-fold), decreased MMP-3 and -8, and had no effect on the remaining MMPs (Fig. 1A, left axis). The exception was MMP-9, whose release was strongly stimulated by both LDLs (Fig. 1A, right axis). In this respect, we observed that LDL(+) and LDL(−) promoted an increased release of MMP-9 compared to untreated control cells of 3.1-fold and 13.8-fold, respectively. Accordingly, LDL(−) stimulated a statistically significant higher release of MMP-9 compared to LDL(+) (4.4-fold, $p = 0.0006$) (Fig. 1A).

Regarding TIMPs, TIMP-3 and TIMP-4 levels were very close to the detection limit of the technique and were not suitably quantified. TIMP-1 levels in serum-free culture media from THP1-CD14 cells were higher in LDL(+)- and LDL(−)-treated cells compared to untreated cells (1.8-fold and 3.7-fold, respectively) (Fig. 1B), and TIMP-1 concentration was significantly higher in monocytes stimulated with LDL(−) than in those treated with LDL(+) (2.1-fold, $p = 0.005$). No effect mediated by LDLs was observed on TIMP-2 release (Fig. 1B).

3.2. Effect of SDX on LDL(−)-induced release of MMP-9 and TIMP-1

On the basis of the known interaction of LDL(−) with a large number of glycosaminoglycans [19,32], as well as due to the ability of glycosaminoglycans to affect the inflammatory and proteolytic signaling cascade in monocytic cells, this project also aimed at investigating the possible effects of SDX in modulating MMP release promoted by LDL(−). First, the authors of this study evaluated the effect of SDX alone compared to untreated THP1-CD14 cells. According to previous reports from this group of researchers, the authors used a concentration of 0.12 LSU/mL, a routine dose pharmacologically circulating in the blood of patients, which was also able to inhibit the MMP-9 release induced by LPS in U-937 and THP1 monocytic cells [22,33]. It was observed that SDX alone had a negligible effect on most MMPs, including MMP-9; only MMP-1 and MMP-8 release were slightly decreased by SDX (Fig. 2A). Regarding TIMPs, SDX slightly increased the release of TIMP-1 (1.1-fold, $p < 0.01$) and had no effect on TIMP-2 (Fig. 2B).

As reported in Fig. 3A, the release of MMP-9 induced by LDL(−) was significantly lower after the addition of SDX compared to LDL(−) alone (decrease of 20.4%, $p < 0.01$, Fig. 3A). A similar effect of SDX was observed in LDL(−)-induced release of MMP-1 and MMP-12 (Fig. 3B and C). TIMP-1 levels induced by LDL(−) in culture media were decreased by SDX compared to cells stimulated with LDL(−) alone (decrease of 37.6%, $p < 0.05$) (Fig. 3D). SDX had no effect on the release of MMP-1, MMP-9, MMP-12, or TIMP-1 induced by LDL(+) (data not shown).

3.3. Modulation of MMP-9 gelatinolytic activity by LDL(−) and SDX

The hyper-expression and release of both MMP-9 and its inhibitor TIMP-1 cast doubts on the net effect that LDL(−)-induced stimulation has on the gelatinolytic activity of THP1-CD14 cells. The zymographic profile of supernatants from the culture media of cells stimulated with LDL(+) and LDL(−), in the presence or absence of SDX (Fig. 4), revealed that untreated THP1-CD14 cells (lane 2) were characterized by two faint gelatinolytic bands corresponding to pro-MMP-2 (72 kDa) and monomeric pro-MMP-9 (92 kDa). A similar pattern was observed in serum-free culture media from SDX-treated cells (lane 3). On the other hand, as highlighted in lanes 4 and 5, LDL treatments induced a significant increase in gelatinolytic activity of the monomeric pro-MMP-9 (asterisk) both in LDL(+) (7.8-fold, $p < 0.05$) and LDL(−) (14.9-fold, $p < 0.01$) treated-THP1-CD14 cells compared to untreated control cells. For the first time, we identified in conditioned media of THP1-CD14 cells additional gelatinolytic bands of approximately 130, 160 and 180 kDa only after LDL(−) treatment (arrow heads, lane 5 and 7) and not observed with LDL(+) stimulation. Furthermore, a gelatinolytic band of about 210 kDa (hashtag, lane 7) is significantly enhanced after SDX and LDL(−) co-treatment, compared to the very faint band of this MMP-9 aggregate/complex found in LDL(−) treatment (lane 5). Studies are *in itinere* for characterizing these unprecedented and novel/intriguing gelatinase isoforms. Pro-MMP-2 proteolytic activity did not differ from that of control cells in any treatment condition.

SDX treatment was able to reduce the monomeric pro-MMP-9

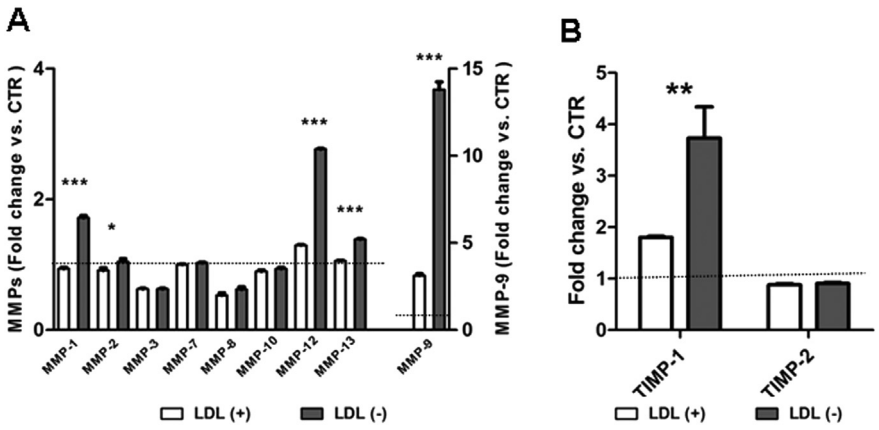

Fig. 1. Levels of MMP (A) and TIMP (B) in LDL(+)- and LDL(−)-treated THP1-CD14 cells. MMPs and TIMPs levels have been normalized to the levels measured in untreated cells. Results are expressed as mean ± SD of the fold change vs. untreated control cells (LDL(+) vs LDL(−) treated THP1-CD14 cells). The results are representative of data obtained from at least 3 independent experiments. Dotted lines indicate the value of untreated cells. *p < 0.05; **p < 0.01; ***p < 0.001.

gelatinolytic activity (lane 7, a decrease of 49%) induced by LDL(−) compared to LDL(−) alone (lane 7 vs lane 5). On the other hand, no statistically significant difference in gelatinolytic activity has been observed when comparing LDL(+) alone with LDL(+) incubated with SDX (lane 6 vs lane 4).

3.4. The role of the CD14 pathway on LDL(−)-induced MMP-9 and TIMP-1 release

To test the role of CD14 in LDL(−)-induced signaling for MMP and TIMP release, experiments were also conducted in THP1 cells not overexpressing CD14 (Fig. 5). In these cells, the extra-cellular secretion of MMP-9 and TIMP-1 was much lower than that observed in THP1-CD14 cells, both in untreated cells (MMP-9: 4.7-fold lower; TIMP-1: 2.6-fold lower) and in LDL-stimulated cells (MMP-9: 14-fold lower for LDL(−) and 3.7-fold lower for LDL(+); TIMP-1: 11.2-fold lower for LDL(−) and 6.5-fold lower for LDL(+)). Moreover, for THP1 cells not over expressing CD14, no difference was observed between LDLs (Fig. 5). Therefore, CD14 appears to be a key to triggering the signaling pathway that leads to the release of MMP-9 and TIMP-1 induced by LDL(−).

3.5. Uptake of LDLs by THP1 and THP1-CD14 cells: Effect of SDX

Analysis with DiI-labeled LDLs demonstrated that the uptake of LDL(−) was higher than that of LDL(+) in THP1-CD14 cells (Fig. 6A). This increased uptake was abolished in the presence of SDX. In contrast, in THP1 cells not over expressing CD14, the uptake of LDL(+) and LDL(−) was similar (Fig. 6B); consequently, SDX had no effect. These observations were confirmed by fluorescence microscopy (inserts in Fig. 6).

3.6. SDX binds to LDL(−)

To understand the mechanism by which SDX interferes in the uptake of LDL(−) by THP1-CD14 cells, the authors of this paper studied the interaction between LDLs and SDX in microtiter wells. Fig. 7 shows the binding of LDL(+) and LDL(−) to SDX coated to microtiter plates (50 mU SDX/well). The binding of LDL(−) to SDX-coated wells was concentration-dependent and increased with increasing incubation time (Fig. 7). In contrast, the binding of LDL(+) to SDX was altered neither by its concentration nor by the incubation time. This observation suggests that the binding of LDL(+) to SDX is non-specific, whereas LDL(−) binds to SDX in a concentration- and time-dependent manner. The amount of LDL(−) bound to SDX was much higher than the amount of LDL(+) in all the conditions, being 11-fold higher than that of LDL(+) at 200 nmol/well and 24 h of incubation.

4. Discussion

Atherosclerotic lesions are the basis for the onset of a wide number of cardiovascular and cerebrovascular clinical manifestations that together represent the leading cause of mortality worldwide, including coronary heart disease, cerebrovascular stroke, renovascular hypertension, and peripheral arterial disease. During the last decades novel risk factors, such as inflammatory processes and lipoprotein oxidative modifications have emerged alongside classical causal factors (including hypertension, dyslipidemia, hypercholesterolemia, diabetes mellitus, obesity, decreased physical exercise, and smoking). In this respect, growing evidence has proposed the involvement of LDL(−) in atherosclerotic plaque formation and evolution [5,7,10,34–38] and in the onset of acute vascular events [39,40]. However, despite several studies providing intriguing hypotheses [6,39,41], to date, the potential

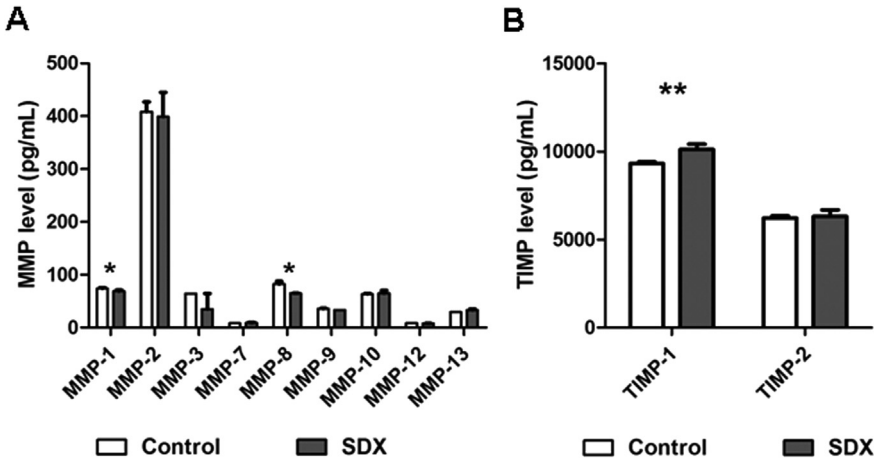

Fig. 2. Effect of SDX on MMP and TIMP release in unstimulated monocytes. Levels of MMPs (A) and TIMP-1 and -2 (B) released by monocytes treated with SDX for 24 h. MMP and TIMP concentrations are expressed as the mean ± SD. The results are representative of data obtained from at least 3 independent experiments. *p < 0.05; **p < 0.01.

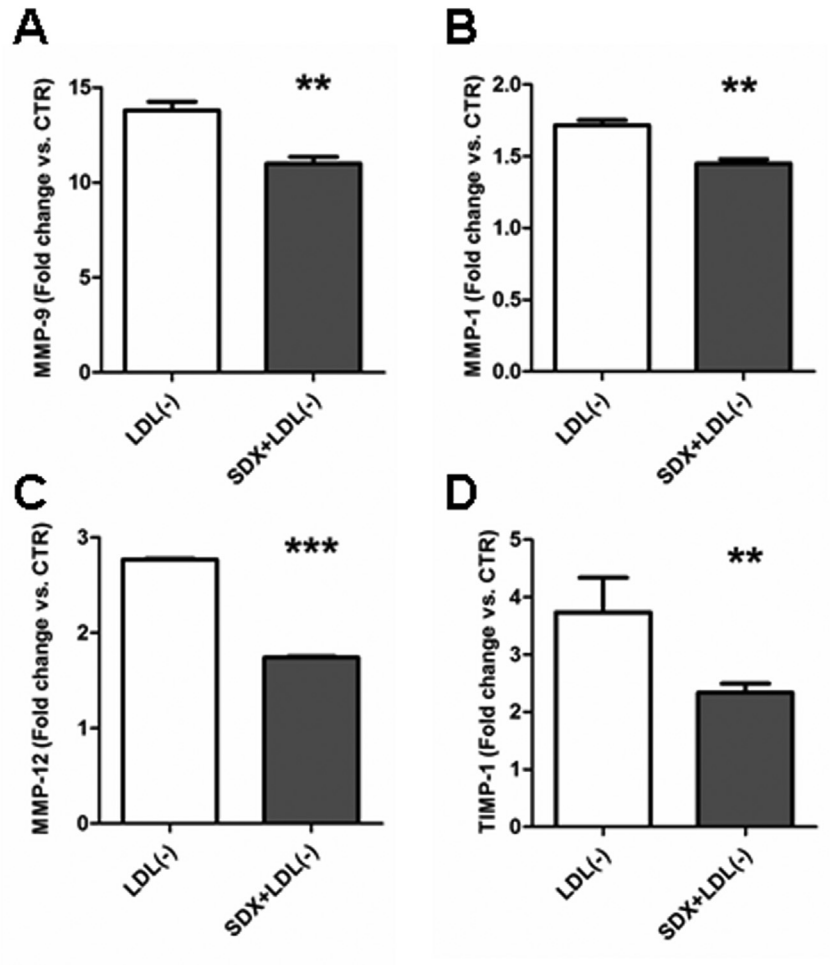

Fig. 3. Effect of SDX on MMPs and TIMP stimulated by LDL(−) in THP1-CD14 cells. Levels of MMP-9, -1, -12 and TIMP-1 released by THP1-CD14 cells treated with LDL(−), with or without SDX for 24 h. MMP and TIMP levels have been normalized to the levels measured in control cells, and results are expressed as fold change vs. control cells (mean ± SD). The results are representative of data obtained from at least 3 independent experiments. **$p < 0.01$; ***$p < 0.001$.

involvement of LDL(−) in lesion remodeling and plaque rupture has not been reported.

One of the factors affecting the instability of atherosclerotic plaque is the imbalance in MMPs and their inhibitors. In this context, this study focused on the ability of LDL(−) to promote MMP and TIMP release from human monocytes. In particular, it has been observed that levels of MMP-9 released in culture media from LDL(−)-stimulated THP1-CD14 cells were significantly higher both in comparison with control cells and with LDL(+)-stimulated monocytes. Surprisingly, TIMP-1 levels also increased after LDL(−) stimulation. Thus, LDL(−) was found to be able to enhance the release of both MMP-9 and its inhibitor, with the fold change ratio of MMP-9 /TIMP-1 indicating a predominance of MMP-9 activity. This predominance was also confirmed by increased gelatinolytic activity determined by zymography in the culture media of cells incubated with LDL(−). LDL(−) treatment was linked to the appearance of novel additional gelatinolytic bands of approximately 130, 160 and 180 kDa, never found neither in untreated cells nor in LDL(+) stimulated monocytes. Some of them were previously described as MMP-9 homodimers or gelatinase isoforms complexed with different glycosaminoglycans and proteoglycans [42,43]. TIMP-1 release promoted by LDL(−) is in agreement with previous findings reporting that increased levels of TIMP-1 in atherosclerotic plaque may be associated with arterial calcification [44] or may play a stabilizing and protective role [1,3]. Another possible explanation relies on cellular compensatory mechanisms aimed at counteracting, with TIMP-1 release, the excessive proteolysis mediated by MMP-9. On the other hand, although reported in other disease models, TIMP secretion is regulated by TLR activation [45], which could explain the ability of LDL(−) to promote its release. Collectively, these results support the multifaceted effects of LDL(−) in triggering proteolytic pathways in monocytes during atherosclerosis.

In contrast to the study authors' present findings in monocytes, it was reported that L5 (the most electronegative subfraction in LDL(−)) inhibited MMP-2 and MMP-9 secretion in endothelial cells [17]. L5 is only present in the blood of hypercholesterolemics, poorly-controlled diabetics, or subjects with acute coronary or ischemic stroke syndromes [37]. This discrepancy could be related both to the differing natures of LDL(−) and L5 and to the different cell types, since each of the LDL subfractions uses different signaling pathways in monocytes and endothelial cells. LDL(−) interacts with the CD14-TLR4 complex in monocytes, whereas L5 activates endothelial cells through LOX-1 binding [46]. On the other hand, oxidized LDL has been shown to induce MMP-2 and MMP-9 expression in activated monocytes [47], an effect that would be presumably mediated by the binding of oxLDL to scavenger receptors of type A. Overall, these different findings highlight the complex regulation of proteolytic pathways by different modified lipoproteins and the distinct cell-type specific effects, and they establish the need for further research to gain insights into these mechanisms in different cell types.

Comparison with control cells indicated that LDL(+) was also able to stimulate the release of MMP-9 from monocytes, despite the fact that these levels were lower than those observed in LDL(−)-stimulated monocytes. This finding is in agreement with a recent paper by Estruch

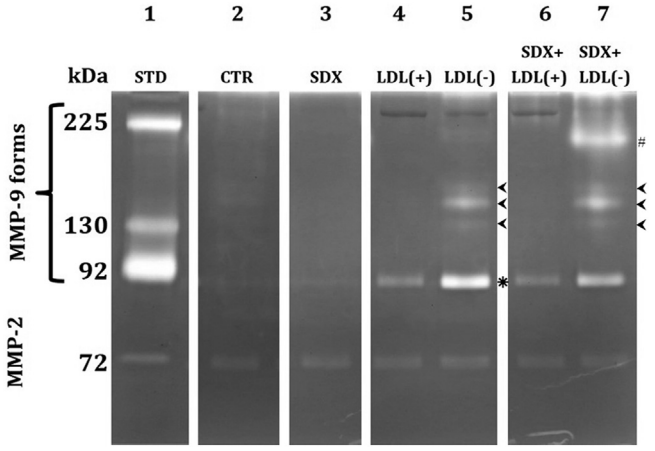

Fig. 4. Zymography. Zymographic profiles of gelatinase MMP-2 and MMP-9 forms released in cell culture supernatants from THP1-CD14 monocytes treated with LDL(+), LDL(−), and/or SDX. Lane 1, STD: Molecular weight standard from peripheral blood; lane 2, CTR: untreated cells; lane 3, SDX: SDX-treated cells; lane 4, LDL(+): electropositive LDL; lane 5, LDL(−): electronegative LDL; lane 6, SDX + LDL(+): co-treatment SDX and electropositive LDL; lane 7, SDX + LDL(−): co-treatment SDX and electronegative LDL. Arrow heads indicate the novel gelatinolytic bands (approximately 130, 160 and 180 kDa) observed only after LDL(−) treatment. Asterisk highlights the monomeric form of MMP-9 at 92 kDa. Hashtag indicates the aggregate/complexed form of MMP-9 at about 210 kDa, found only in SDX and LDL(−) co-treatment. Zymogram was performed by loading the same volume of supernatants. The shown zymogram is representative of at least 5 independent experiments.

et al., which reported that LDL(+) and LDL(−) interact with TLR, activating the same intracellular pathways, but the effect induced by LDL(+) is much lower than that of LDL(−) [12].

The lack of effect of LDL(−) in THP1 cells not over expressing CD14 clearly indicates the involvement of CD14 in the MMP-9 and TIMP-1 release induced by LDL(−). These results are in agreement with the ability of LDL(−) to activate the CD14-TLR4 signaling pathway, thereby leading to the activation of the same inflammatory pathways promoted by lipopolysaccharide (LPS) [9]. The stimulation of the CD14-TLR4 pathway in monocytes is considered a crucial step for monocyte activation and cytokine release. Once activated, CD14-TLR4 induces in monocytes a cascade of events that finally culminates in inflammatory processes mediated by the release of cytokines, such as MCP-1, IL-8, IL-6, IL-1β, and IL-10 [9,12]. Similarly, the stimulation of TLR signaling promotes the activation of transcription factors (e.g., NF-kB, AP-1) and kinases (p38 MAPK) involved in the regulation of MMP synthesis and release [48–50]. Furthermore, inflammatory mediators secreted by activated monocytes could also affect MMP release [51], resulting in the activation of a vicious cycle, in which MMP secretion is promoted both directly by LDL(−) recognition of CD14-TLR4 and indirectly by the pro-inflammatory cytokines released after such activation.

The involvement of CD14 in LDL(−)-induced gelatinolytic activity is also supported by the higher uptake found in THP1-CD14 than in THP1 cells and the inhibitory effect of SDX. This higher uptake could be attributed to the higher activation of TLR4, a co-receptor that associates with CD14. Although TLRs are usually considered signaling receptors, these receptors have also been involved in mediating the internalization of lipoproteins or other ligands, such as LPS. In this regard, TLR4 has been described as essential for oxidized LDL-induced lipid accumulation in macrophages [52] and probably facilitates the foam cell transformation of these cells. In addition, TLR4 can mediate not only oxidized LDL but also native LDL uptake by macropinocytosis [53]. Some studies also show that LPS can be endocytosed through TLR4 in monocytes [54], and this process is regulated by CD14.

The present study also shows that SDX, a highly purified mixture of

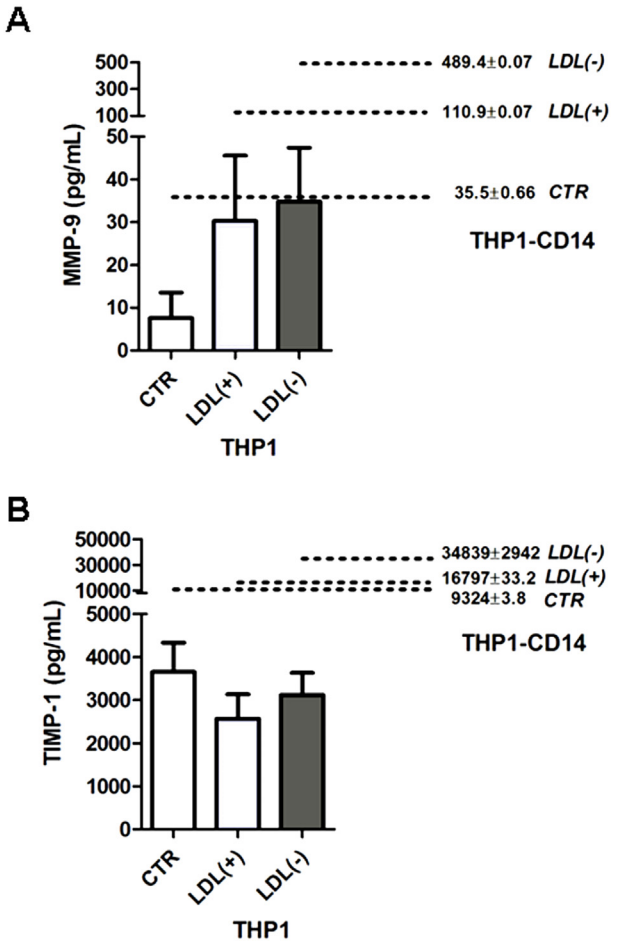

Fig. 5. Role of TLR4-CD14 signaling pathway. Effect of LDLs on the release of MMP-9 and TIMP-1 in THP1 cells not over expressing CD14. Levels of MMP-9 (A) and TIMP-1 (B) released by THP1 treated with LDLs for 24 h. MMP and TIMP concentrations are expressed as the mean ± SEM. The results are representative of data obtained from at least 3 independent experiments. Horizontal dotted line indicates the values detected in THP1-CD14 cells (right side).

GAG composed of 80% fast-moving heparin and 20% dermatan sulfate, can inhibit the release of several MMPs and TIMPs, including MMP-9 and TIMP-1. SDX is a widely used anti-thrombotic drug that has also been shown to have anti-inflammatory and hypolipemic properties. The findings of the present study suggest that SDX may also represent a putative drug targeting the proteolytic pathways activated by LDL(−) and a possible anti-atherosclerotic agent [20]. In this context, an interesting case report highlighted a reduction of carotid plaque after SDX treatment [55]. Our group previously demonstrated that SDX modulates both inflammatory response [23] and MMP release [22] in LPS-stimulated monocytic cell lines. However, this is the first report regarding the ability of SDX to counteract the inflammatory and proteolytic processes induced by LDL(−) in monocytes. Herein, we observed that MMP-9 and TIMP-1 levels, as well as gelatinolytic activity, were significantly lower after combined treatment of THP1-CD14 monocytes with SDX and LDL(−) than after LDL(−) treatment alone. Noteworthy, we evidenced that glycosaminoglycan SDX down-regulated the gelatinolytic activity of the monomeric form of MMP-9 (92 kDa) in LDL(−)-treated monocytes. Moreover, only in the SDX and LDL(−) co-treatment, we found the appearance of a novel gelatinolytic band at about 210 kDa, which could represent a MMP-9 heterodimer complex, in agreement with high molecular weight MMP-9 forms found previously with different glycosaminoglycans and proteoglycans [42,43]. These complexes have altered biochemical properties compared with

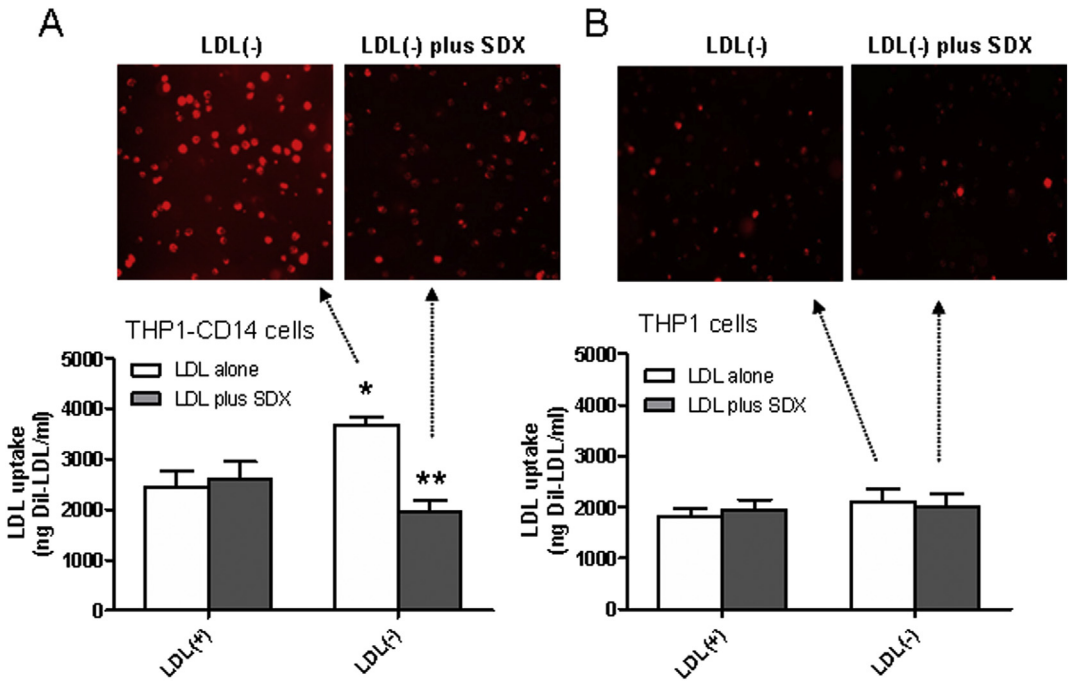

Fig. 6. Uptake of LDL subfractions to THP1-CD14 and THP1 cells. The uptake of LDL subfractions to cultured cells was quantified by measuring the amount of fluorescence bound to cells after lipid extraction, as described in Methods. Panel A shows data obtained in THP1-CD14 cells and panel B data obtained in THP1 cells. Fluorescence is expressed as the mean ± SD from 6 independent experiments. Inserts are fluorescence micrographs obtained as described in Methods. *p < 0.05 vs LDL(+); **p < 0.05 vs LDL alone.

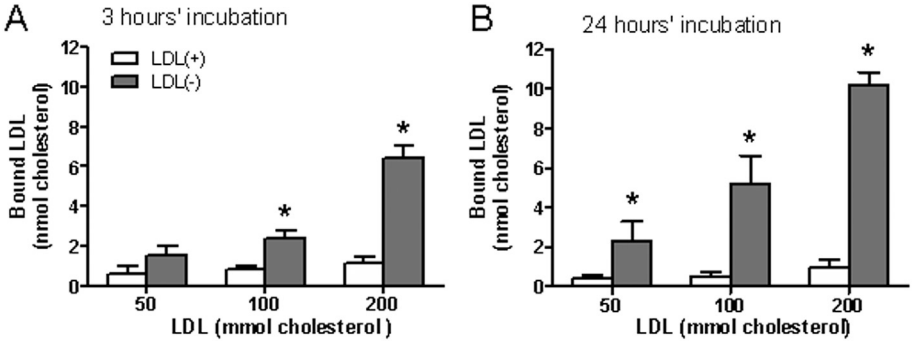

Fig. 7. Binding of LDL subfractions to Sulodexide. The binding of LDL subfractions to SDX-coated microwells was quantified by measuring the amount of bound cholesterol, as described in Methods. Panel A shows data after 3 h of incubation and panel B data after 24 h of incubation. Cholesterol concentration is expressed as the mean ± SD from 5 independent experiments. *p < 0.05 vs LDL(+).

the MMP-9 monomer, such as weaker affinity to gelatin [43]. The affinity of LDL(−) for GAGs may represent the biochemical mechanism underlying the effects of SDX in reducing the release of MMP from LDL (−)-stimulated monocytes.

It has been previously documented that, despite both molecules presenting a net negative charge, LDL(−) is able to interact with glycosaminoglycan molecules, mainly with those composing artery proteoglycans [19,56]. In the present study we demonstrate that SDX interacts with LDL(−), but not with LDL(+), in a concentration- and time- dependent manner. In this perspective, SDX may act through different hypothetical mechanisms: firstly, as a sequestrating agent, able to prevent the interaction of LDL(−) with CD14, and, alternatively, could also interact with CD14, blocking the recognition of LDL (−). Further investigations are underway to clarify the possible mechanism(s) of SDX-CD-14 binding.

The possibility of discovering novel biomarkers for atherosclerotic plaque initiation and progression, as well as identifying potential therapeutic targets, represents a demanding task for improving diagnosis and therapy for cardiovascular diseases. In this respect, LDL(−) is an emerging risk factor for the development of atherosclerotic lesions; however, the biochemical and molecular mechanisms activated by LDL (−) have yet to be fully understood. The results herein described provide further evidence of the involvement of LDL(−) in driving the proteolytic processes related to atherosclerosis, in particular in promoting the release of specific MMP and TIMP from human monocytes. Furthermore, this study provided novel findings about the ability of sulodexide to counteract proteolytic activities induced by LDL(−) in human monocytes, suggesting how this glycosaminoglycan could be a potential anti-atherosclerotic treatment.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Disclosures

The authors declare that they have no conflicts of interest.

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