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Pro-fibrotic effect of oxidized LDL in cardiac myofibroblasts

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ABSTRACT

Inflammatory signals associated with cardiac diseases trigger trans-differentiation of cardiac fibroblasts to cardiac myofibroblasts. Cardiac myofibroblasts are the main cell type involved in the development of cardiac fibrosis, a diffuse and disproportionate accumulation of collagen in the myocardium. Although the role of the scavenger like-lectin receptor LOX-1 was previously investigated in cardiac fibroblasts and fibrosis, the involvement of the LOX-1 ligand -oxidized low-density lipoprotein (oxLDL)- on cardiac myofibroblast function still remains unexplored. In the present work, we investigated the effect of oxLDL/LOX-1 on fibrotic markers and cardiac myofibroblast function. Our *in vitro* results showed that oxLDL increased cardiac myofibroblast proliferation, triggered an increase in the synthesis of collagen type I and fibronectin containing extra domain A, and stimulated collagen type I secretion. oxLDL also decreased cardiac myofibroblast migration, collagen gel contraction and cell area, without modifying α-smooth muscle actin protein levels. These effects were dependent on LOX-1, because LOX-1 knockdown abolished oxLDL effects. Collectively these data showed that oxLDL has important modulatory effects on cardiac myofibroblast function.

1. Introduction

Cardiac fibrosis is an important global health problem associated with the pathological remodeling of the heart [1]. This process is characterized by an increment in extracellular matrix (ECM) deposition, which leads to stiffness, arrhythmia and heart failure, with systolic and/or diastolic dysfunction [2]. The inflammatory milieu associated with cardiac diseases triggers trans-differentiation of cardiac fibroblasts (CFs) to cardiac myofibroblasts (CMFs) [1]. CMFs are characterized by the expression of α-smooth muscle actin (α-SMA) and extracellular matrix protein hypersecretion such as collagen I, collagen III and fibronectin [3].

High cholesterol plasma levels are a risk factor for cardiovascular diseases. Cholesterol is transported in the plasma associated with lipoproteins, including the low-density lipoprotein (LDL). The oxidized form of LDL (oxLDL) is the main cholesterol lipoprotein responsible for cardiovascular pathologies. Increased oxLDL plasma levels are found in patients with heart failure [4–6] and in the ventricles of patients with different coronary artery diseases [7]. The principal receptor for oxLDL is LOX-1, a scavenger like-lectin receptor encoded by the *orl1* gene [8]. LOX-1 is expressed in several cell types [9–11] including CFs [12]. The LOX-1 canonical signaling pathway involves NADPH oxidase 2 (NOX2) activation and reactive oxygen species production [8]. This receptor has been implicated in the development of cardiac fibrosis [13,14]. LOX-1 knock out reduces ECM accumulation in the heart upon angiotensin II infusion [15] and decreases collagen accumulation and cardiac hypertrophy triggered by chronic ischemia [14]. Moreover, LOX-1 is important in CF growth [14] and its levels decrease during CF senescence [16]. However, the role of LOX-1 in CMF, the main cell involved in cardiac fibrosis remains almost unknown.

In this work, we investigated the effect of oxLDL/LOX-1 on fibrotic markers and CMF. Our results showed that oxLDL exacerbates the CMF pro-fibrotic phenotype by activation and auto-induction of the LOX-1 receptor.

Abbreviations: α-SMA, α-smooth muscle actin; CFs, cardiac fibroblasts; CMFs, cardiac myofibroblasts; COL1, collagen type I; ECM, extracellular matrix; Fn-EDA, fibronectin containing extra domain A; oxLDL, oxidized low-density lipoprotein.

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2. Materials and methods

Cardiac myofibroblast culture. CFs were isolated from the left ventricle of 2-month-old Sprague–Dawley rats, as we previously described [17]. For differentiation to CMFs, CFs were cultured in DMEM/F12 medium, supplemented with 2% SFB and 10 μg/mL TGF-β1 (Merck Millipore) for 96 h [17]. For all experiments, cells were treated with oxLDL (10 nM) during different times. oxLDL was obtained as described [18].

Western blot. CMFs were lysed in RIPA buffer 1X, supplemented

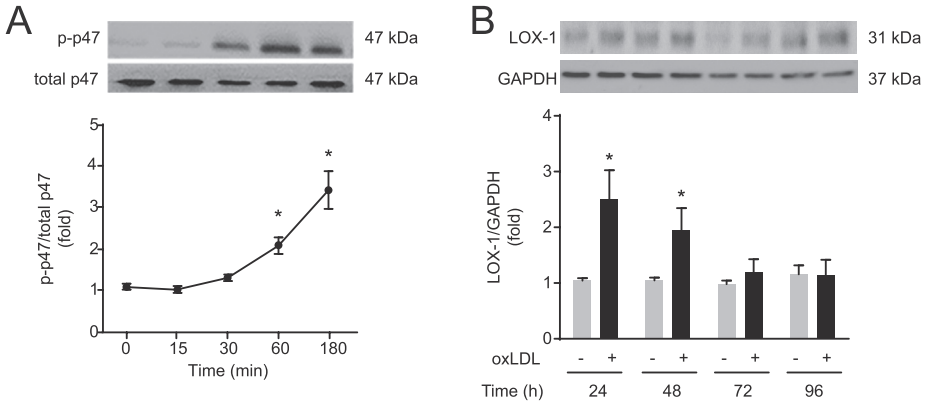

Fig. 1. oxLDL activates LOX-1 in CMF. **(A)** CMFs were treated for 0, 15, 30, 60, and 180 min with oxLDL (10 μg/mL). Protein extracts were obtained and total (total p47) and phosphorylated p47 (phospho-p47) levels were determined by western blotting. Data is shown as a representative immunoblot with the analysis of the phospho-p47/total p47 ratio. Data are the mean ± SEM (n: 4 independent experiments). Statistical analysis was performed with one-way ANOVA and Dunnet post-test. *p < 0.05 vs 0 min time point. **(B)** CMFs were treated during 24, 48, 72 and 96 h with oxLDL (10 μg/mL). LOX-1 protein levels were determined in protein extracts by Western blot. GADPH was used as a loading control. Data is displayed as a representative immunoblot with the analysis of the LOX-1/GAPDH ratio. Data are the mean ± SEM (n: 4 independent experiments). Statistical analysis was performed using the Student *t*-test. *p < 0.05 vs the respective control.

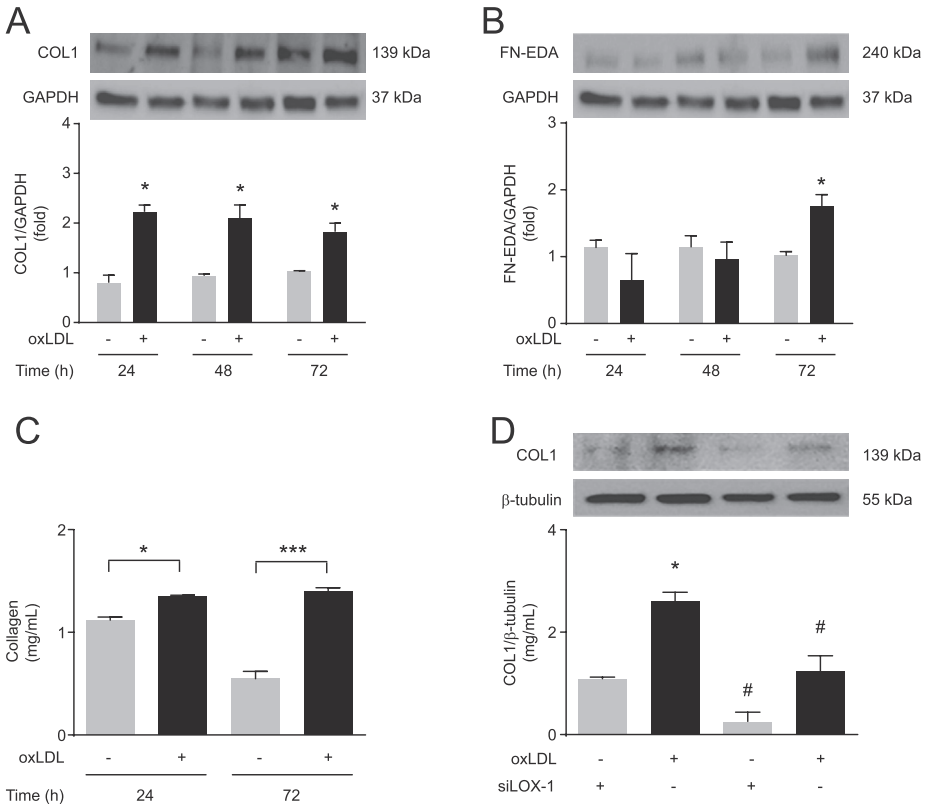

Fig. 2. oxLDL increases synthesis and secretion of collagen type I and fibronectin-EDA. CMFs were treated during 24, 48 and 72 h with oxLDL (10 μg/mL) and protein extracts were obtained. **(A)** Collagen type I (COL1) and **(B)** Fibronectin-EDA (Fn-EDA) protein levels were determined by Western blot. GADPH was used as a loading control. Data is displayed as a representative immunoblot with the respective analysis of the COL1/GAPDH and Fn-EDA/GAPDH ratios. Data are the mean ± SEM (n: 4 independent experiments). Statistical analysis was performed with the Student *t*-test. *p < 0.05 vs respective control. **(C)** CMFs were treated for 24 and 72 h with oxLDL (10 μg/mL). Once culture supernatants were collected, collagen levels were determined by Sirius red staining. Data are the mean ± SEM of 4 independent experiments. Statistical analysis was performed with the Student *t*-test. *p < 0.05 and ***p < 0.001 vs respective controls. **(D)** CMFs were treated with LOX-1 siRNA or scrambled siRNA (control) and incubated for 18 h. The cells were then treated with oxLDL (10 μg/mL) for 24 h. COL1 was determined by Western blot. β-tubulin was used as a loading control. Data is shown as a representative immunoblot with the respective analysis of the COL1/β-tubulin ratio. Data are the mean ± SEM of 4 independent experiments. Statistical analysis was performed with one-way ANOVA, with the Dunnet post-test. *p < 0.05 vs control; #p < 0.05 vs oxLDL.

with inhibitor cocktail (Roche LifeScience). Lysates were centrifuged at 14,000 rpm for 15 min and total protein content was determined using the micro BCA protein assay (Thermo Fisher Scientific). Equivalent amounts of protein were loaded on SDS-polyacrylamide gels. Proteins were then transferred to PDVF membranes, which were blocked with 5% fat-free milk (w/v) in TBS-Tween 0.1% for 1 h at room temperature. Membranes were probed overnight at 4 °C with the following primary antibodies: anti-GAPDH (1:5,000, SC-32233 Santa Cruz Biotechnology Inc.), anti-LOX-1 (1:2,500, AB60178 Abcam), anti-phospho-p47 (1:1000, SC-86190 Santa Cruz Biotechnology Inc.), anti-total p47 (1:1,000, SC-74514 Santa Cruz Biotechnology Inc.), anti-Fn-EDA (1:1,000, AB6328 Abcam), anti-COL1 (1:2000, AB34710 Abcam), and anti-α-SMA (1:6,000, AB7817 Abcam). Membranes were then incubated with peroxidase-conjugated secondary antibodies for 1 h at room temperature. Finally, the ECL Western blotting Detection Kit (Biological Industries) and a Syngene XT4 equipment were used for detection. Densitometric analysis was performed using UN-SCAN-IT gel 6.1 and normalized to the corresponding GAPDH levels.

Immunofluorescence assay. CMFs (3 x 10^4) cultured on sterilized glass coverslips were fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.1% Triton X-100 for 15 min at room temperature. After blocking with 3% bovine serum albumin for 60 min at room temperature, cells were incubated with anti-Ki67 (1:100, #9129 Cell Signaling Technology Inc.) or anti-αSMA (1:1,000, A5228, Merck), overnight at 4 °C and then with Alexa fluor 488-conjugated secondary antibodies (1:1,000, A32731, Thermo Fisher Scientific) for 2 h at room temperature. Nuclei were stained using Hoechst (1:5000, Thermo Fisher Scientific) and coverslips were mounted using DAKO (Agilent Technologies). Images were obtained using a Carl Zeiss Pascal 5 confocal microscope [19].

Cell proliferation assay. CMF proliferation was determined using three techniques: i) Trypan blue assay: cells were detached with trypsin 1X and counted by the trypan blue exclusion method on a Neubauer chamber [19]; ii) MTT assay: cells were incubated with 12 mM MTT in DMEM/F12 at 37 °C for 4 h. Formazan crystals were solubilized in 100 μL of DMSO and absorbance was measured at 570 nm using a microplate reader; and iii) the Ki67 cell proliferation assay: Ki67 was detected by immunofluorescence, using anti-Ki67 antibodies.

LOX-1 silencing. RNAiMAx (7.5 μL, Thermo Fisher Scientific) plus Opti-MEM® (142.5 μL, Thermo Fisher Scientific) and siRNA LOX-1 (Mission siRNA LOX1, SASI_Rn01_00106751 and SASI_Rn02_00267601, Merck) dissolved in Opti-MEM® (150 μL) were prepared at room temperature by mixing. Cells were incubated with 300 μL of the mix at 37 °C. After 16 h, the medium was changed to DMEM/F12, 2% FBS, and cells were incubated for 24 h. Scrambled siRNA (Silencer® Negative Control, Merck) was used as a negative control.

Collagen gel contraction assay. CMFs were seeded (2 x 10^5), cultured and treated with or without oxLDL. After 24 h, cells were detached with trypsin and 100,000 cells were mixed with 200 μL of rat tail collagen type I solution (3.63 mg/mL, Santa Cruz Biotechnology Inc.) and DMEM/F12 containing 10 mM Hepes buffer (GIBCO, Grand Island, NY) in a total volume of 1 mL. The mix was incubated in a 24-well plate for 2 h at 37 °C for collagen polymerization. The gels were then detached from the wells. Gel contraction was determined after 48 h and expressed as the ratio between the initial and the final area of collagen gel for each treatment.

Wound healing assay. Confluent CMF cultures were maintained

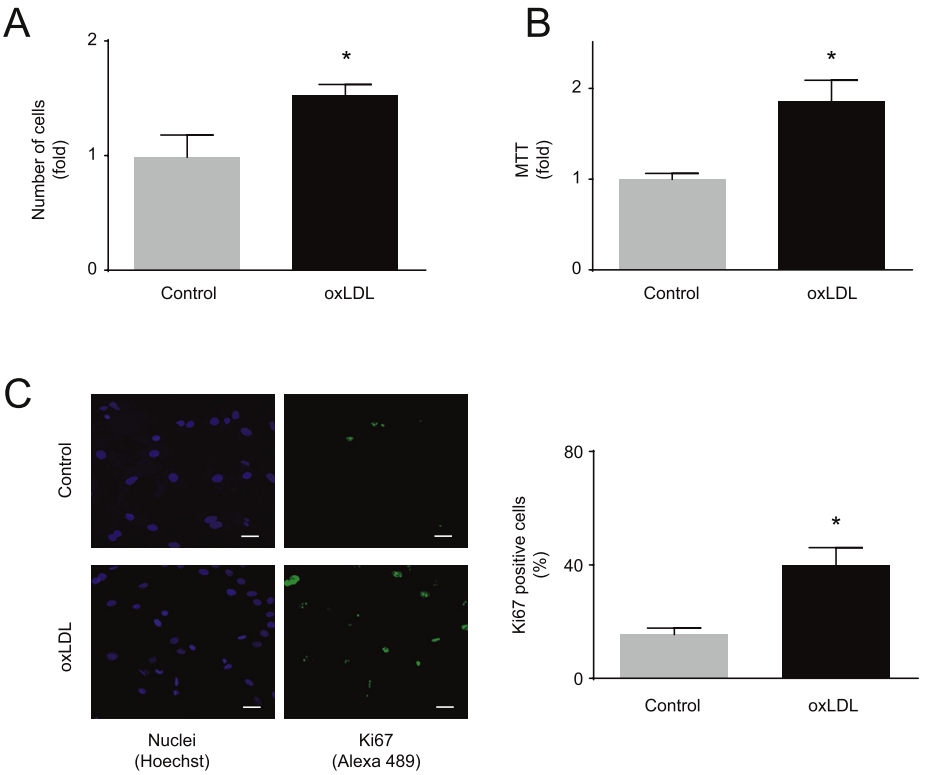

Fig. 3. oxLDL induces CMF proliferation. CMFs were treated with oxLDL (10 μg/mL) for 72 h. **(A)** Cell number was determined in a Neubauer chamber using the Trypan blue assay. **(B)** Cell number was determined with the MTT assay. **(c)** Ki67 positive cells were determined by immunofluorescence using anti-Ki67 primary antibodies and revealed with Alexa 489 secondary antibodies (green). Nuclei were stained using Hoechst (blue). Data show a representative confocal microscopy image with the respective quantification as percentage of Ki67-positive cells. Scale bar = 20 μm. Data are the mean ± SEM of 5 independent experiments. Statistical analysis was performed with the Student *t*-test. *$p < 0.05$ vs the respective control. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

in serum free DMEM/F12 medium, supplemented with bromodeoxyuridine (10 μM) for 24 h before the experiments. Then, a wound was made with a yellow tip. Images were acquired at 0 and 24 h using a Nikon Eclipse TS100 microscope, equipped with a 519CU 5.0M CMOS Camera. Images were analyzed using the Image J program (NIH) and data were expressed as percent of wound area reduction.

Statistical analysis. Data are expressed as mean ± SEM of n independent experiments. Statistical analysis was performed by one-way ANOVA, followed by the Dunnet post-test, using the GraphPad Prism Software. $p < 0.05$ was considered significant.

3. Results

oxLDL activates and increases LOX-1 levels in CMFs. In order to assess the functionality of LOX-1, CMFs were stimulated with oxLDL and we evaluated p47 phosphorylation, which is a NOX-2 activation marker. Treatment of CMFs with oxLDL induced p47 phosphorylation at 60 min, and these levels were maintained at 180 min (Fig. 1A). Moreover, LOX-1 protein levels increased at 24 and 48 h of treatment with oxLDL (Fig. 1B). These results suggest that oxLDL triggers LOX-1 in CMFs, increasing LOX-1 protein levels.

oxLDL increases ECM synthesis and secretion in CMFs. The effect of oxLDL on ECM protein synthesis was evaluated by measuring collagen type I (COL1) and fibronectin containing extra domain A (Fn-EDA). oxLDL induced an increase in COL1 protein levels from 24 to 72 h (Fig. 2A). oxLDL significantly increased Fn-EDA protein levels only at 72 h (Fig. 2B). Extracellular COL1, as a measure of COL1 secretion, was increased at 24 and 72 h of treatment (Fig. 2C). Silencing of LOX-1 decreased oxLDL-induced COL1 synthesis (Fig. 2D). These results suggest that oxLDL triggers LOX-1 in CMFs, increasing COL1 and Fn-EDA synthesis and secretion.

oxLDL induces CMF proliferation. oxLDL induced CMF proliferation, as determined by cell number counting (Fig. 3A). This result was confirmed with the MTT assay (Fig. 3B) and by evaluating the proliferation marker Ki67 (Fig. 3C).

oxLDL decreases CMF migration and contractility. According to the wound healing assay, treatment of CMF with oxLDL for 24 h decreased migration (Fig. 4A). Moreover, oxLDL also reduced collagen gel contractibility at both 24 and 72 h (Fig. 4B). Silencing of LOX-1 prevented oxLDL-induced reduction of collagen gel contractibility (Fig. 4B). Interestingly, these changes were associated with a decrease in cell area (Fig. 4C), without modification of α-SMA protein levels (Fig. 4D).

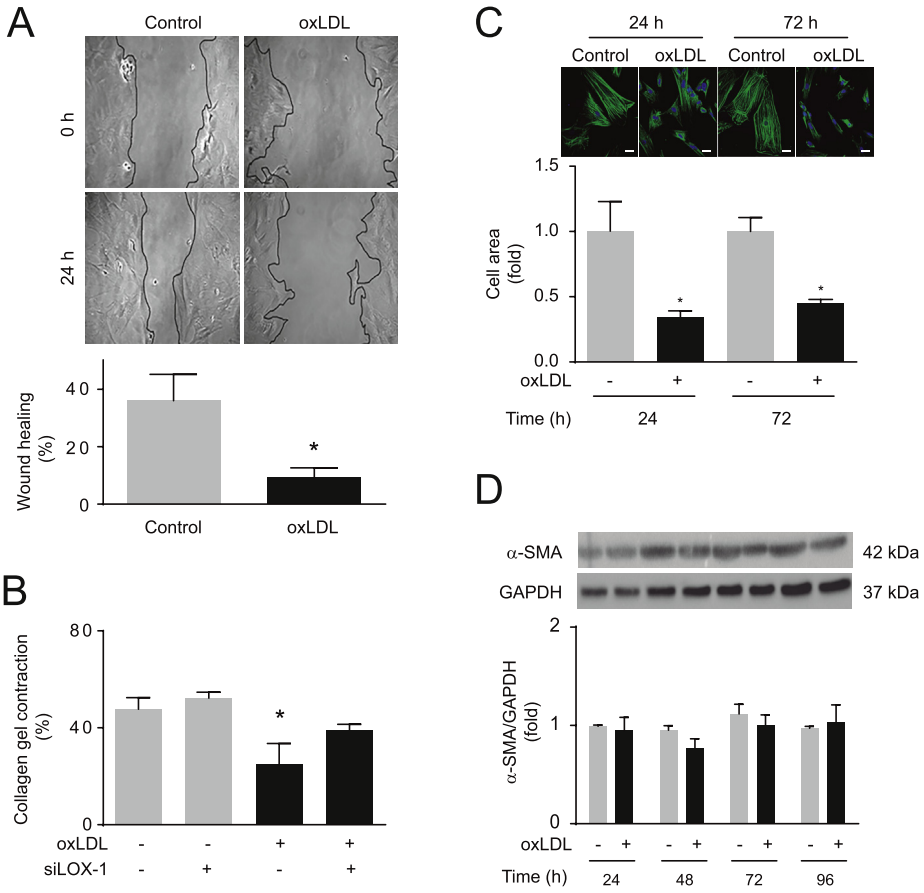

Fig. 4. oxLDL changes functional characteristics of CMFs. **(A)** CMF migration was assessed using the wound healing assay. **(B)** Collagen gel contraction assay. CMFs were treated with LOX-1 siRNA or scrambled siRNA (control) and incubated for 18 h. The cells were then treated with oxLDL (10 μg/mL) for 24 h to perform the collagen gel contraction assay. **(C)** CMFs were treated with oxLDL (10 μg/mL) for 72 h and then analyzed by immunofluorescence using anti-α-smooth muscle actin (α-SMA) antibodies and revealed with Alexa 489 secondary antibodies (green). Nuclei were stained using Hoechst (blue). Data shows a representative confocal microscopy image with the respective quantification as cell area relative to control. Scale bar = 20 μm. **(D)** CMFs were treated during 24, 48, 72 and 96 h with oxLDL (10 μg/mL). Protein extracts were obtained and α-SMA protein levels were determined by Western blot. GADPH was used as a loading control. Data is displayed as a representative immunoblot with the analysis of the α-SMA/GAPDH ratio. Data are the mean ± SEM of 5 independent experiments. Statistical analysis in **(A)** and **(C)** was performed with the Student *t*-test. *$p < 0.05$ vs the respective control. Statistical analysis in **(B)** was performed with one-way ANOVA, with the Dunnet post-test. *$p < 0.05$ vs control. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

The main findings of this study showed that oxLDL triggers an increase of ECM synthesis and secretion, associated with increased CMF proliferation. However, we also observed a decrease in CMF migration, collagen gel contraction and cell area, without modification in α-SMA protein levels. These effects were dependent on LOX-1 because LOX-1 knockdown abolished oxLDL effects.

LOX-1 has been previously associated with cardiac fibrosis. LOX-1 knock out mice showed reduced cardiac fibrosis, triggered by myocardial infarction or sustained hypertension [14,15]. Previous evidence has indicated that LOX-1 knock out fibroblasts show decreased growth and synthesis of collagen [13,14]. Here, we depicted that LOX-1, also regulates collagen synthesis and proliferation in CMF. We found that activation of LOX-1 by oxLDL induced COL1 synthesis and secretion and increased CMF proliferation. Because the conversion of CF to CMF is recognized as a critical event in the genesis and progression of cardiac fibrosis [20], the role of LOX-1 on CMF could be crucial in the development of this process. In this context, the induction of CMF proliferation could be relevant as a mechanism for perpetuation of cardiac fibrosis.

oxLDL plasma levels are an important risk factor for cardiovascular diseases [6] and constitute a prognostic indicator of mortality in patients with congestive heart failure [21]. ox-LDL plasma levels correlate with a lower ejection fraction and increased severity of clinical symptoms [21,22]. Moreover, ox-LDL levels in patients submitted to cardiovascular surgery are higher in left ventricular blood than in peripheral blood [23], suggesting that oxLDL could have more important effects on the heart that in other organs. Our results showed that oxLDL, through LOX-1, activates NOX2 and increases LOX-1 protein levels in CMFs. Therefore, increased left ventricular blood levels of oxLDL could potentiate LOX-1 profibrotic actions in the heart, particularly in CMFs.

Treatment of CMFs with oxLDL induced a decrease in cell area, collagen gel contractile capacity and cell migration. These results could be explained by CMF dedifferentiation to CF. However, since we did not observe a decrease in α-SMA levels, these effects would not be associated to dedifferentiation. Wang et al. have described that LOX-1 is important for the maintenance of the cytoskeleton [16]. Overexpression of LOX-1 in senescent CFs resulted in cytoskeleton reorganization and increased proliferation rate [16]. Although we did not evaluate cytoskeleton organization, the reduction of cell area suggests a mayor restructuration of the cytoskeleton in CMFs. On the other hand, reduced collagen gel contractile capacity and cell migration could decrease the reparative capacity of CMFs after a heart injury. This result could explain the association between increased oxLDL plasma levels with decreased ejection fraction and increased severity of clinical symptoms after myocardial infarction [15,24,25]. However, further work is required to assess whether oxLDL increased levels after heart injuries affect scar formation and tissue repairing.

In conclusion, oxLDL, though a LOX-1 dependent mechanism, increased CMF proliferation and synthesis and secretion of COL1 and Fn-EDA. However, CMF migration, collagen gel contraction and cell area decreased, without a reduction of α-SMA levels. These data showed that oxLDL has important modulatory effects on CMF function, suggesting an important role of oxLDL in several heart diseases, through the regulation of CMF function.

Declaration of competing interest

All authors have declared that there are no conflicts of interest.

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