# Effect of myeloperoxidase modified LDL on bovine and human aortic endothelial cells

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**Abstract.** Cardiovascular disease associated with atherosclerosis is a leading cause of death worldwide. Atherosclerosis is primarily caused by the dysfunction of vascular endothelial cells and the subendothelial accumulation of oxidized forms of low-density lipoproteins (LDL). Early observations have associated fibrin deposition with atheroma plaque formation, which has led to the proposition that a decrease in endothelial cell fibrinolysis may negatively influence atherogenesis. It has been recently demonstrated that myeloperoxidase modified LDL (MoxLDL) decreases endothelial cell profibrinolytic capacity in real-time. The present study investigated the role of MoxLDL in endothelial cell dysfunction by determining the molecules that may be involved in decreasing the fibrinolysis of human aortic endothelial cells (HAEC). Accordingly, reverse transcription-quantitative PCR was performed to screen for the differential expression of major genes that are implicated in the fibrinolytic process. In addition, the response of the latter cell type to MoxLDL was compared with bovine aortic endothelial (BAE) cells. Furthermore, the effect of the treatment on the generation of reactive oxygen species (ROS) was also determined. Although the current study did not demonstrate an association between MoxLDL treatment and a change in the expression of any major fibrinolytic factor in HAEC, a discrepancy between HAEC and BAE cells with respect to their response to modified LDL treatment was observed. The result have also demonstrated that MoxLDL does not increase ROS generation in HAEC as opposed to the other major type of modified LDL, cupper oxidized LDL (CuoxLDL) that was reported to exhibit a positive effect at this level. The present study provided important insight into the different effects of MoxLDL and CuoxLDL in endothelial cells, which may aid future studies to determine the various signaling pathways that are promoted by these molecules. The results of the present study may be utilized to identify potential molecular drug targets for the treatment of atherosclerosis.

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## Introduction

Cardiovascular diseases (CVDs) are a wide spectrum of diseases affecting the heart and blood vessels. This spectrum includes mainly coronary heart disease (CHD) and cerebrovascular disease (1). Atherosclerosis, the culprit cause of myocardial and cerebral infarction, is the principle agent of mortality and morbidity worldwide (2). Atherosclerosis is a cardiovascular disease marked by the dysfunction of the endothelium, formation of lipid-laden plaques, and narrowing and hardening of the blood vessels. Over the centuries, many hypotheses were postulated to explain the mechanism behind the initiation and development of the atherosclerotic lesions. The three main theories are the lipid theory, the oxidation hypothesis of atherogenesis and the response-to-injury inflammatory hypothesis. The cornerstone focus of the oxidative hypothesis is that specifically oxidized low density lipoproteins (LDL) generated by myeloperoxidase pathways are injurious to the arterial cell wall as reported by Chilsom and colleagues in 1979. The inflammatory response-to-injury hypothesis is regarded as the refinement of the previous theory; during the 19th century, the pathologist Rudolf Virchow described atherosclerosis as a chronic inflammation (3). This hypothesis proposes that injury to the endothelium and its dysfunction, which leads to fibrin deposition, are the initiating events along with its increased permeability to modified lipoproteins (4). Oxidation of low density lipoproteins have been of major interest since Steinberg *et al* showed that native LDL does not accumulate in macrophages whereas modified lipoproteins do (5).

Oxidized LDL (oxLDL) unlike the native LDL (nLDL) was shown to initiate and trigger the inflammatory process of the disease (6). Several candidates were then proposed to elucidate LDL modification including myeloperoxidase (MPO). Ample studies showed the responsibility of MPO in atherogenesis

in humans. Immunohistochemical and biochemical analyses conducted by Daugherty *et al* (7) co-localized MPO and its products within human atheromatous plaques (7-9). On the same note, it has been shown that individuals with a deficiency in the MPO enzyme are less prone to develop CVDs on the long term. Another relationship appears in which increased systematic levels of MPO indicates the presence of coronary artery disease (CAD) (10). A major function of the vascular endothelium is the fine-tuning of the delicate components of the coagulation and fibrinolytic systems. It maintains antico-agulant and antithrombotic environment by releasing a variety of molecules that regulate blood hemostasis assuring a profibri-nolytic state (11). Accordingly, the endothelium secretes major fibrinolytic factors including tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA), and plasminogen activator inhibitor-1 (PAI-1) and express specific receptors that binds these factors supporting a fibrinolytic environment (12). Also, as mentioned above, early observations have correlated fibrin deposition with atheroma plaque formation. This led to the proposition that a decrease in fibrinolysis in endothelial cells may negatively influence atherogenesis. In parallel, recent studies have also revealed that patients with atherosclerosis exhibited a hypofibrinolytic phenotype (13). Since it has been previously confirmed that MoxLDL decreases EA.hy926 profibrinolytic capacity in real-time without delineating the mechanisms by which this modified LDL can alter pericellular fibrinolysis (14), we tried in the present study to perform a preliminary dissection of the molecules that might be involved in decreasing fibrinolysis by using primary HAEC as a model. The study also included an ephemeral comparison regarding the disparate effect of MoxLDL on two different primary cultures of endothelial cells, bovine aortic endothelial cells and human aortic endothelial cells, as well as its effect on reactive oxygen species (ROS) generation in the latter model.

## Materials and methods

*Cell culture*. BAE cells were cultured in Dulbecco's Modified Eagle's Medium-AQ (DMEM-AQ, Sigma Aldrich) supplemented with 10% heat inactivated fetal bovine serum (FBS; Sigma Aldrich), and 1% penicillin/streptomycin mixture. HAEC's were cultured in EBM-2 Basal Medium supplemented with human epidermal growth factor (hEGF), vascular endothelial growth factor (VEGF), R3-insulin-like growth factor-1 (R3-IGF-1), ascorbic acid, hydrocortisone, human fibroblast growth factor-beta (hFGF-β), FBS, and gentamicin/amphotericin-B (GA) (Lonza). Cells were maintained at 37°C in a humidified 5% $CO_2$ incubator. BAE cells and HAEC were used between passages 5-9.

*In vitro treatment of BAE and HAEC*. BAE cells and HAEC were seeded in 6-well plates at a density of 5x10^5 cells/well in triplicates. Cells were either left untreated or treated with nLDL (100 $\mu$g/ml) or with MoxLDL (25, 50, or 100 $\mu$g/ml) (15,16). Cell morphology was monitored after treatment and images (x200 magnification) were captured using a phase contrast inverted microscope (Leica).

*Recombinant MPO preparation*. Recombinant MPO was prepared as described previously (14). Briefly, in order to

express MPO, a recombinant plasmid that codes for prepro-myeloperoxidase was constructed and named pNIV2703. This plasmid contains an MPO fragment coding for amino acid 11 in the putative signal sequence to amino acid 696. The pNIV2703 expression vector was transfected into CHO cells by electopermeabilization. Cell supernatants were recovered to assay the production level and the enzymatic activity of secreted molecules. Each batch solution was characterized by its activity (U/ml), protein concentration (mg/ml), and specific activity. Peroxidative activity was determined using *o*-dianiside as the substrate. Protein concentration was measured using the Lowry assay, with ovalbumin as a standard. Each batch was checked for endotoxin using the endotoxin detection kit (Lonza). Concentration was always less than 100 pg/ml, which, taking into account the final dilution of the MPO-treated LDL fraction, would contribute a final concentration of less than 0.1 pg/ml to the MoxLDL supplemented medium added to the cells.

*Isolation of nLDL and MoxLDL preparation*. nLDL was isolated and MoxLDL prepared as previously described (14); lipoprotein particles were isolated from plasma from sterile blood pouches using density-gradient ultracentrifugation. The nLDL fraction (*d*=1.019-1.063) was stored under nitrogen at 4°C in the dark and oxidized according to the procedure described below: Prior to oxidation, nLDL was gel filtered (PD-10 column, Pharmacia) and 1.6 mg of native LDL was oxidized by 2.1 chlorinating units of recombinant MPO, to generate MoxLDL in the presence of 1 mM $H_2O_2$ in 2 ml phosphate buffered saline (PBS) at pH 6.5 for 5 min. LDLs were desalted again after MPO treatment. Protein concentration was measured by the Lowry assay, using ovalbumin as protein standard.

*RNA extraction from BAE cells and HAEC*. Cells were treated as indicated above and then harvested for total RNA extraction. RNA was extracted using the RNeasy plus mini kit (Qiagen) according to manufacturer's instructions. RNA extracts from different samples were analyzed by spectrophotometry. Absorbance values (A) were recorded at two wavelengths, (260 and 280 nm) to assess purity (A260/A280) and measure the concentration (A260) of extracted RNA.

*Reverse transcription of RNA*. Extracted RNA was reverse transcribed to a complementary DNA (cDNA) using QuantiTect reverse transcription kit according to manufacturer's instructions (Qiagen). Briefly, genomic DNA (gDNA) contaminants were eliminated by incubating extracted RNA in gDNA wipeout buffer (Qiagen) at 42°C for 2 min. Then reverse transcription was performed by incubating samples with master mix (reverse transcriptase (RT), RT primer mix, and RT buffer; Qiagen) at 42°C for 15 min and later inactivated at 95°C for 3 min.

*Reverse transcription-quantitative PCR (RT-qPCR)*. RT-qPCR was performed using SYBR-Green (Qiagen), on a Real-time Systems (Bio-Rad). 20 $\mu$l of reaction was added in each well containing: 1 $\mu$g cDNA, 1 $\mu$l of 10 $\mu$M forward and reverse primers (Table I; Sigma), and 10 $\mu$l Sybr Green. The cycling conditions were: 95°C for 3 min, 40 cycles of 95°C for 10 s,

Table I. Primer sequences for the genes of interest.

| Gene | Forward | Reverse |
|---|---|---|
| tPA | 5'-CCGGCTACGGCAAGCA-3' | 5'-TGGATGGGTACAGTCTGACATGA-3' |
| uPA | 5'-CCGCTTTCTTGCTGGTTGTC-3' | 5'-TATTGTCGTTCGCCCTGGTG-3' |
| uPAR | 5'-GGTGACGCCTTCAGCATGA-3' | 5'-CCCACTGCGGTACTGGACAT-3' |
| tPAR | 5'-TGGATGGGAGACAATCTGTA-3' | 5'-TGCCCTCGATTAAAGTCTTG-3' |
| PAI-1 | 5'-CAGACCAAGAGCCTCTCCAC-3' | 5'-ATCACTTGGCCCATGAAAAG-3' |
| FXIII A1 | 5'-CCAGATTGACTTCAACCGTCCC-3' | 5'-GACACCAGCAAAAACCCAACACTGG-3' |
| GAPDH | 5'-AAATCCCATCACCATCTTCC-3' | 5'-TCACACCCATGACGAACA-3' |

tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor; tPAR, tissue plasminogen activator receptor; PAI-1, plasminogen activator inhibitor-1; FXIII A1, fibrin-stabilizing factor.

and 60°C for 30 s. Each reaction was performed in triplicate and GAPDH was used as a reference gene for normalization. Relative gene expression levels and resulting fold changes were calculated using the comparative $2^{-\Delta\Delta Cq}$ method (17). Primers sequences for genes of interest were selected from the RTPrimerDB online real-time PCR primer database (rtprimerdb.org) and verified using the online NCBI BLAST tool.

*Analysis of BAE cell viability.* Propidium iodide (PI) cell viability assay was used to assess the viability of BAE cells following treatment as indicated above. BAE cells were washed once with PBS and detached by incubation with accutase cell detachment solution (Thermo) at 37°C for 2 min. Both detached and floating cells were then collected and resuspended in 1X binding buffer solution (BD Biosciences) and stained with PI (BD Biosciences) for 15 min at room temperature in the dark. Samples were run on a FACSCalibur flow cytometer (BD Biosciences) and data were analyzed using CellQuest Pro software version 5.1 (BD Biosciences). BAE cells were identified by their forward-scatter (FSC) and side-scatter (SSC) properties. Viable and dead cell populations were identified as PI- and PI+ cells, respectively (18-20). A total of 10,000 single cell events were measured for each sample.

*Measurement of ROS generation by HAEC.* ROS production by HAEC was assessed following treatment as indicated above using the radical-sensitive fluorescent probe, 2,7-dichlorodi-hydrofluorescein diacetate ($H_2DCFDA$; Molecular Probes). HAEC were washed once with PBS and detached by incubation with accutase cell detachment solution at 37°C for 2 min. Cells were then incubated with H2DCFDA (10 $\mu$mol/l) for 45 min at 37°C in a humidified 5% $CO_2$ incubator, and washed twice with PBS. Cells were finally resuspended in PBS and acquired using FACSCalibur flow cytometer. The experiment was performed in triplicates and $H_2O_2$ (1 and 10 $\mu$g/ml) was used as a positive inducer of ROS. Intracellular ROS levels, reflected by the mean fluorescence intensity (MFI) of 2,7-dichlorofluorescein (DCF)-stained HAEC were measured using CellQuest Pro software. HAEC were identified based on their FSC and SSC characteristics.

*Statistics.* GraphPad Prism software (version 6) was used to perform statistical data analysis and drawing of graphs. Data

are presented as mean ± standard error of the mean (SEM). The non-parametric Kruskal-Wallis test followed by a Dunn's multiple comparison post-hoc test was performed to determine statistical differences among the different experimental groups. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Expression of selected fibrinolytic genes in HAEC in response to nLDL or MoxLDL treatment.* A previous study has demonstrated that MoxLDL delays fibrinolysis pericellularly in EA.hy926 endothelial cells (14). Several genes such as tPA, uPA, tPA receptor (tPAR), uPA receptor (uPAR) and PAI-1 are known to be key players in the process of fibrinolysis (21). However, whether their expression levels in HAEC are altered upon MoxLDL treatment remains unknown. Therefore, we have assessed by RT-qPCR the mRNA expression profile of the above-mentioned genes in HAEC treated with nLDL or MoxLDL for 24 h. Fibrinolysis activators, tPA and uPA, in addition to their corresponding receptors, tPAR and uPAR, did not show a significant variation in their mRNA expression levels following treatment with nLDL or MoxLDL. PAI-1 also, the major plasminogen activator inhibitor, did not show a variation in expression (Fig. 1). Finally, FXIII, a principal hemostatic factor and the protein responsible for crosslinking fibrin meshwork was found not to be expressed in HAEC (data not shown).

*Effect of nLDL or MoxLDL treatment on the morphology and viability of HAEC.* HAEC were cultured in a 6-well plate and were left untreated or treated with either 100 $\mu$g/ml of nLDL or MoxLDL for 24 h. The cells were then visualized under an inverted phase contrast microscope. HAEC showed a healthy morphology with no or little detached cells detected (Fig. 2). Supplementary PI staining and FACS analysis were also carried out and did not show any significant effect of MoxLDL on HAEC viability (data not shown).

*Effect of nLDL and MoxLDL treatment on the morphology and adherence of BAE cells.* In order to study the effect of nLDL and MoxLDL on BAE cells, confluent monolayer cells were treated with 100 $\mu$g/ml of nLDL, and 25, 50, and 100 $\mu$g/ml of MoxLDL for 24 h. Following MoxLDL treatment, a significant
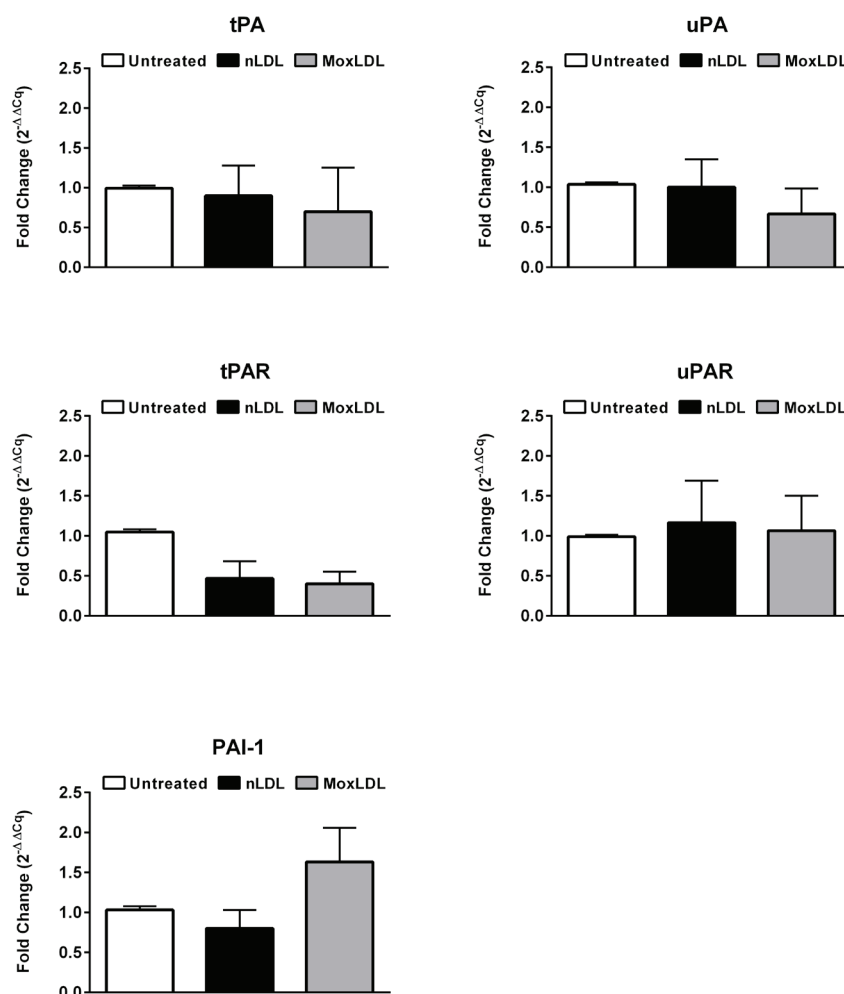
Figure 1. Relative Gene Expression of Fibrinolysis Mediators. tPA, uPA, tPAR, uPAR and PAI-1 levels in human aortic endothelial cells were determined via reverse transcription-quantitative PCR following treatment with physiological concentrations (100 $\mu$g/ml) of nLDL or MoxLDL for 24 h. Data are presented as the mean ± SEM (n=3) fold change in mRNA expression vs. control expression (untreated cells). tPA, tissue plasminogen activator; urokinase plasminogen activator; tPAR, tPA receptor; uPAR, uPA receptor; PAI-1, plasminogen activator inhibitor-1; nLDL, native low-density lipoprotein; MoxLDL, myeloperoxidase modified low-density lipoprotein.
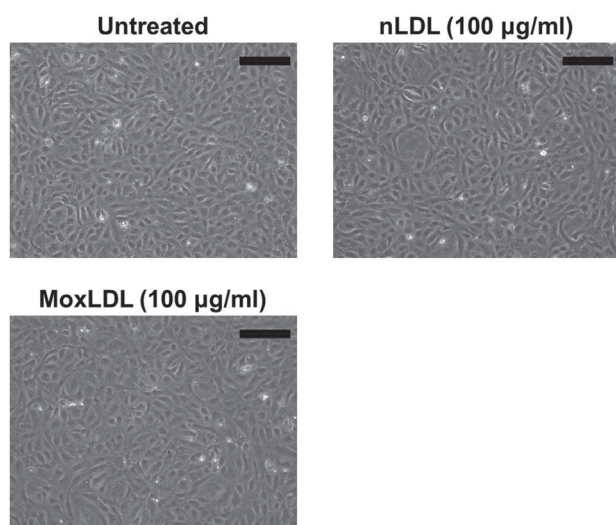


Figure 2. Morphology of HAECs following treatment with nLDL or MoxLDL. HAEC (passage 5-6) were cultured in 6-well plates and left untreated or treated with 100 $\mu$g/ml nLDL or MoxLDL for 24 h (Scale bars, 100 $\mu$m). HAECs, human aortic endothelial cells; nLDL, native low-density lipoprotein; MoxLDL, myeloperoxidase modified low-density lipoprotein.

high percentage of detached and floating cells was noted when BAE cells were visualized under an inverted phase contrast microscope. However, nLDL treatment did not induce BAE cell detachment and cells were maintained as a monolayer (Fig. 3).

*Effect of nLDL and MoxLDL treatment on the viability of BAE cells.* We assumed that the detachment of BAE cells might be a consequence of a cytotoxic effect induced by MoxLDL treatment. Therefore, PI viability assay was performed in order to assess the cytotoxic effect of MoxLDL as well as the effect of nLDL on BAE cells. Treated BAE cells were harvested, stained with PI, and analyzed by flow cytometry. As expected, untreated cells and nLDL-treated cells showed some little extent of spontaneous cell death. However, adequate cell viability analysis of MoxLDL-treated BAE cells was not possible due to that fact that cell fragments and debris compromised the bulk of the culture (Fig. 4).

*Effect of nLDL or MoxLDL treatment on ROS production by HAEC.* OxLDL has been previously reported, to increase ROS production by endothelial cells (22). Therefore, the ability of
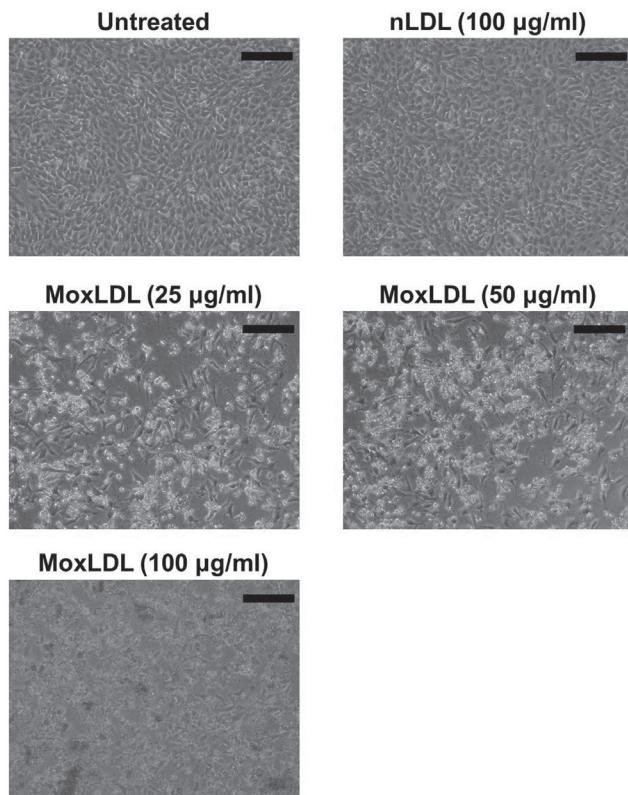
Figure 3. Effect of nLDL or MoxLDL treatment on the morphology and adherence of BAE cells. BAE cells (passage 8-9) were cultured in 12-well plates and left untreated or treated with 100 µg/ml nLDL or with 25, 50 and 100 µg/ml MoxLDL for 24 h (scale bars, 100 µm). nLDL, native low-density lipoprotein; MoxLDL, myeloperoxidase modified low-density lipoprotein; BAE, bovine aortic endothelial.

MoxLDL to induce endothelial cells to generate ROS was assessed by H2DCFDA staining combined with flow cytometry analysis. MoxLDL treatment resulted with no increase but in a decrease in the production of ROS as compared to untreated cells; however, this decrease did not attain statistical significance (P>0.05; Fig. 5). Likewise, nLDL treatment showed a non-significant (P>0.05) decrease in ROS production (Fig. 5). $H_2O_2$ (1 and 10 µg/ml), used as a positive control, induced high and significant levels of ROS by HAEC (Fig. 5).

## Discussion

In our study, the interaction between MoxLDL, endothelial cells, and mainly fibrinolysis was investigated. All previous research documented and explored the signaling pathway by which CuoxLDL initiate inflammation and subsequent atherogenesis (23). On the contrary, very little is known about the MoxLDL. MPO modified LDL is the more physiologically relevant model of LDL oxidation due to the fact that immuno-histochemical analyses conducted by Daugherty et al (7) and others co-localize MPO and some modified amino acids in the ApoB100 moiety of LDL, such as chlorotyrosine or nitrotyrosine, within human atheromatous plaques (7-9,24). Therefore, in our study, we aimed to examine the effect of MoxLDL on different cell models: BAE and HAEC.

BAE are primary cells derived from the aorta of cows. Previous research performed on these cells showed that 150 µg/ml of MoxLDL treatment for 24 h was markedly cytotoxic as judged by MTT assay (25). However, this study was performed using another oxidation product of MPO, which is peroxinitrite modified LDL. Similarly, our results showed tremendous death and fragmentation of BAE cells following MoxLDL treatment (LDL modified by hypochlorus acid), even at low to normal physiological concentrations (25, 50 and 100 µg/ml) (15). By recurring to PI staining and FACS analysis, we unsuccessfully tried to assess the exact magnitude of MoxLDL's effect in our experimental model and that was due to the remarkable fragmentation of the cells that were difficultly sorted and in a very bad shape. Hence, our results pave the way for future investigation at this level that should be carried out using maybe lower concentrations of modified LDL in order to better characterize the mechanism of cell death. On the same note and in the context of atherosclerosis, little in vivo experimentation was conducted in animals. It was not documented that animals especially bovine develop atherosclerosis naturally. This may be due to the fact of their short life span, in comparison to humans, unique digestive and metabolic characteristics, different diet and cholesterol intake. This raises again an interesting question regarding the modification of LDL molecules in their system and whether it involves similar mechanisms that are already seen and documented in humans.

Contradictory to the effect of MoxLDL on cell death in BAE cells, previous reports confirmed that MoxLDL treatment (up to 100 µg/ml) does not reduce viability or induce cell death in human endothelial cells, human umbilical Vein Endothelial Cells (HUVEC) (26). Preponderance of studies reported that uptake by LOX-1 receptor, a type of scavenger receptors minimally expressed on inactivated vascular endothelium, is involved in endothelial activation, dysfunction and subsequent initiation of atherosclerosis. It has been shown that CuoxLDL binds to LOX-1 receptor increasing transcriptional activation of LOX-1 mRNA synthesis, entering in a positive feedback loop that exacerbate the vascular dysfunction (27,28). Intracellularly, this binding activates membrane bound NADPH oxidase that rapidly elevates the level of ROS by generating super oxide ion, exacerbating the inflammatory signal (29). Enough evidence had been accumulated to state that CuoxLDL stimulates ROS generation; however, very limited research was conducted on endothelial cells using MoxLDL to check the levels of ROS production. As a matter of fact, CuoxLDL is not physiologically relevant. In particular, CuoxLDL is not closely related to the oxidized LDL present in vivo. High concentrations of Cu or Fe used in vitro to oxidized LDL are never met physiologically. Several enzymes were proposed as a physiological alternative to the modification of LDL such as MPO and peroxidasin (16). Additionally, immunohistochemical and biochemical analyses conducted by Delporte et al (8) co-localize MPO and its modified amino acids products, such as chlorotyrosine or nitrotyrosine within human atheromatous plaques (7,9,13,24). Also, high serum levels of MPO are regarded as a risk factor in CADs (30).

Data in the literature show MoxLDL's key involvement in the interplay and fine-tuning between the coagulation and fibrinolytic systems on the endothelial cell surface; yet little is known about the mechanism by which MoxLDL binds to the endothelium initiating the dysfunction. The endothelium
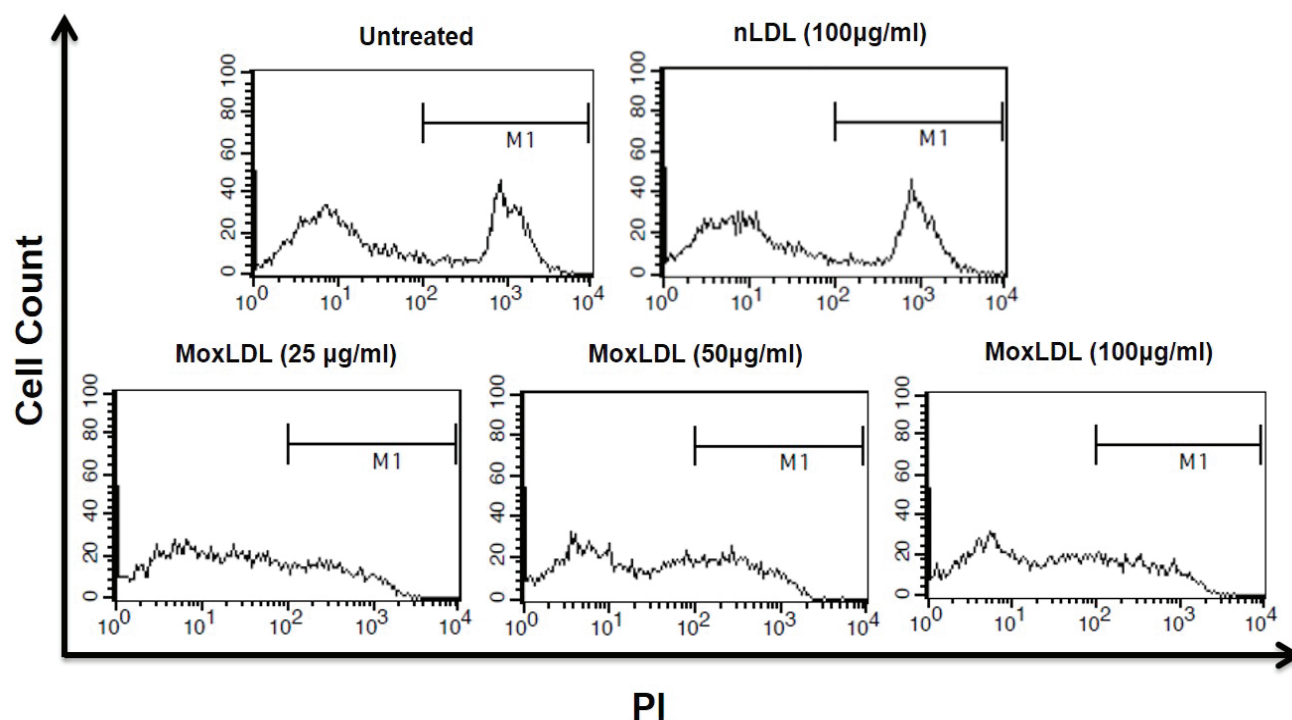
Figure 4. Effect of MoxLDL Treatment on BAE Cell Viability. Representative flow cytometry histogram plots demonstrating propidium iodide (PI) staining of BAE cells that were left untreated or were treated with 100 µg/ml of nLDL or 25, 50, or 100 µg/ml MoxLDL for 24 h. Data are representative of the experiment performed in triplicate.
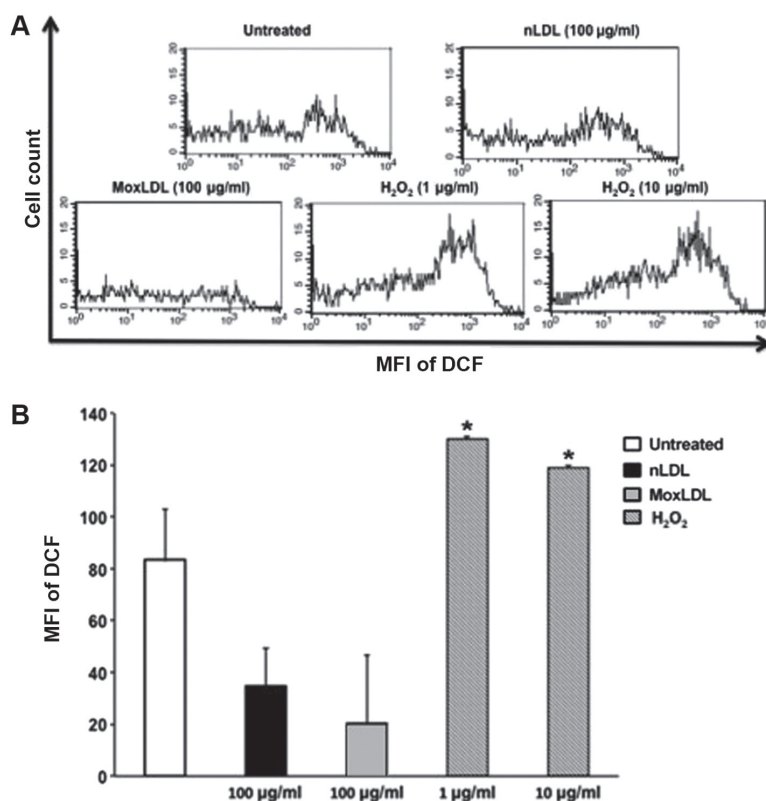


Figure 5. Effect of MoxLDL on HAEC reactive oxygen species production. HAECs (passage 5-6) were cultured in 6-well plates and left untreated or treated with 100 $\mu$g/ml nLDL, MoxLDL or $H_2O_2$ (1 or 10 $\mu$g/ml) for 24 h. Cells were then harvested, labeled with $H_2$DCFDA and analyzed via flow cytometry. (A) Representative histogram plots demonstrating the geometric MFI of DCF-stained HAEC from different conditions. (B) Bar graph presenting the MFI values (mean ± SEM) of DCF-stained HAECs (n=3). *P<0.05 vs. the control. MoxLDL, myeloperoxidase modified low-density lipoprotein; HAECs, human aortic endothelial cell; MFI, mean fluorescent intensity; DCF, 2,7-dichlorofluorescein.

secretes tPA and uPA, and expresses specific receptors that bind these factors supporting a fibrinolytic environment (13). Moreover, the binding of plasminogen and tPA to fibrin or their respective receptors ensures the protection from $\alpha_2$-antiplasmin and $\alpha_2$-macroglobulin inhibition. This means that endothelial cells and their receptors aid and promote pericellular fibrinolysis (31). Early evidence correlates fibrin deposition and plaque formation. Consistent with that, studies documented that fibrin deposition on endothelial cells alters their cobblestone morphology, induce the production of IL-8 and inflammatory and chemotactic molecules, and most importantly renders them more permeable to LDL infiltration (32). Due to previous technical limitations, studying the effect of MoxLDL on pericellular fibrinolysis was a challenge. However, more recently, a technical device that detects fibrinolysis in real-time was successfully created (14). In the latter model, the authors associated MoxLDL with a delayed fibrinolytic capacity of the endothelium, but no effect on PAI-1, tPA, uPAR, tPAR or plasmin inhibitors: $\alpha_2$-antiplasmin and $\alpha_2$-macroglobulin was recorded. These cells that were used in the study were hybrid cells that arise from a fusion between HUVECs and thioguanine-resistant clone of A549 human carcinoma cell line (33). In accordance with this previous finding suggesting the null transcriptional effect of MoxLDL on the fribrinolytic key players, our results verified so while using the role model of cells to study atherosclerosis: Human aortic endothelial cells (HAEC). We additionally studied another potential effector that might be the cause behind this delay: Factor XIII. Factor XIII, also known as fibrin stabilizing factor, crosslinks every E-unit with a D-unit of fibrin monomers further stabilizing the fibrin meshwork. Contradictory observations are published on the fact whether endothelial cells secrete FXIII or not (34). Our results showed that HAEC that were analyzed by RT-qPCR do not express it.

As for the negative effect of MoxLDL on pericellular fibrinolysis in endothelial cells, and since we've shown that it was not related to a change in gene expression, one potential mechanism that remains effective is the possibility of a physical interaction between MoxLDL and specific cell membrane receptors that are expressed on endothelial cells, more specifically tPAR and uPAR receptors, which can explain this delay as a competitive inhibition on the receptor itself by MoxLDL. This was seen with other pro-atherogenic molecules such as apolipoprotein (a) which was shown to bind tPAR receptors with high affinity (35).

Finally, preceding reports stated that MoxLDL lacked an effect on the expression of LOX-1 gene expression as opposed to CuoxLDL that was reported to increase LOX-1 expression (14,36). This was further validated by our experiments that did not show an increase in ROS production intracellulary in HAEC (15). Therefore, it is likely that MoxLDL acts through a still undetermined receptor(s), eliciting signaling transduction pathways that are dissimilar to CuoxLDL.

This study gave interesting insights onto the effect of MoxLDL on the gene expression of central players in fibrinolysis in HAEC as well as a potentially different mechanism of action for MoxLDL as compared to CuoxLDL. In hope that our results will pave the way for more experiments, some prospective research work can be proposed including the investigation of the potential receptor(s) that is/are responsible for binding to MoxLDL. Accordingly, a series of knockdown experiments (tackling potential receptors/signaling proteins) can be conducted in order to study the signaling transduction pathway(s) promoted by MoxLDL in HAEC thus helping us reveal the key players that are responsible for the phenotype that is being uncovered after subjecting the cells to physiological levels of MPO-modified LDL.

## Availability of data and materials

Previously reported [expression of Selected Fibrinolytic Genes in HAEC in Response to nLDL or MoxLDL Treatment] data were used to support this study and are available at [doi: 10.1155/2014/134635-doi: 10.1371/journal.pone.0038810]. These prior studies (and datasets) are cited at relevant places within the text as references [Daher *et al*, 2014-Zouaoui Boudjeltia *et al*, 2012].

## Authors' contributions

LV, KB, MK and JD conceived the current study and designed the experiments. GS and JD wrote the manuscript. JD and SB edited the manuscript. GS and SB performed data analysis.

## Ethics approval and consent to participate

The CHU Charleroi Hospital Ethics Committee (Comité d'Ethique I.S.P.PC: OM008) has approved blood sampling and has specifically approved this study. The studies conform to the principles outlined in the Declaration of Helsinki.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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