Particulate Matter Promotes In Vitro Receptor-Recognizable Low-Density Lipoprotein Oxidation and Dysfunction of Lipid Receptors

Natalia Manzano-León,[1] *Jaime Mas-Oliva,*[2] *Laura Sevilla-Tapia,*[1] *Rocío Morales-Bárcenas,*[1] *Jesús Serrano,*[3] *Marie S. O´Neill,*[4] *Claudia M. García-Cuellar,*[1] *Raúl Quintana,*[1] *Inés Vázquez-López,*[1] *and Álvaro R. Osornio-Vargas*[1,5,]

[1] Instituto Nacional de Cancerología, C.P. 14080 México, D.F., México

[2]Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, C.P. 04510, México, D.F., México

[3]Facultad de Ciencias, Universidad Nacional Autónoma de México, México, D.F., México

[4]Departments of Environmental Health Sciences and Epidemiology, University of Michigan School of Public Health, Ann Arbor, MI 48109, USA

[5]Department of Paediatrics, University of Alberta, Edmonton T6G 1C9, Canada; E-mail: *osornio@ualberta.ca*

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ABSTRACT: Particulate matter may promote cardiovascular disease, possibly as a consequence of its oxidative potential. Studies using susceptible animals indicate that particulate matter aggravates atherosclerosis by increasing lipid/macrophage content in plaques. Macrophage lipid uptake requires oxidized low-density lipoprotein and scavenger receptors; same receptors are involved in particulate matter uptake. We studied in vitro particulate matter potential to oxidize low-density lipoproteins and subsequent cell uptake through scavenger receptors. Particulate matter-induced low-density lipoproteins oxidation was evaluated by the thiobarbituric acid assay. Binding/internalization was tested in wild type and scavenger receptor–transfected Chinese hamster ovary cells, and in RAW264.7 cells using fluorescently labeled low-density lipoproteins. Dose-dependent binding/internalization only occurred in scavenger receptor–transfected Chinese hamster ovary cells and RAW264.7 cells. Competition binding/internalization using particles showed that particulate matter induced decreased binding (∼50%) and internalization (∼70%) of particle-oxidized low-density lipoproteins and native low-density lipoproteins. Results indicate that particulate matter was capable of oxidizing low-density lipoproteins, favoring macrophage internalization, and also altered scavenger and low-density lipoproteins receptor function. © 2012 Wiley Periodicals, Inc. J Biochem Mol Toxicol 27:69–76, 2013; View this article online at wileyonlinelibrary.com. DOI 10:1002/jbt.21452

KEYWORDS: **Particulate Matter; Low-Density Lipoprotein; Scavenger Receptor; Macrophages; Oxidative Potential**

INTRODUCTION

Exposure to particulate matter (PM) in polluted cities represents a significant risk to human health [1]. PM is a complex, dynamic mixture of materials with toxic potential that depends on size and composition [2, 3]. Several studies have linked cardiovascular mortality and complications of preexisting atherosclerotic disease after exposure to PM present in polluted air [4–6].

The mechanisms by which exposure to PM complicates a preexisting cardiovascular condition have not been fully elucidated yet. However, experimental studies in humans and animals have provided a wealth of evidence on how PM exposure can influence relevant cardiovascular processes including increased blood pressure, increased blood viscosity, and altered fibrinogen levels [7]. Arrhythmias, changes in electrocardiographic parameters, and increased vasoconstriction have been also identified as complications in elderly subjects [8] and healthy people occupationally exposed to different types of particles [9, 10]. Subjects

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implanted with defibrillators show an increase in the frequency of defibrillator interventions and an increase in the level of serum C-reactive protein in response to PM exposure [7, 11, 12].

Several studies in humans have also shown a relationship between carotid intimal-medial thickness and exposure to PM less than 2.5 μm in aerodynamic diameter ($PM_{2.5}$), in conjunction with atherosclerosis development and coronary artery calcification [13, 14]. Suwa et al. [15] observed advanced coronary artery and aorta atherosclerotic plaques in Watanabe heritable hyperlipidemic rabbits exposed to PM less than 10 μm in aerodynamic diameter (PM_{10}) compared to unexposed control animals of the same strain. Atherosclerotic plaques in exposed animals had a larger number of cells containing an increased amount of intracellular lipids, resulting in a greater quantity of total lipids in aortic lesions. Moreover, Araujo et al. [16] studied the proatherogenic effects of ultrafine particles and $PM_{2.5}$ in genetically susceptible mice (apolipoprotein $E^{-/-}$), which after exposure to PM presented larger atherosclerotic lesions than control animals. Likewise, apolipoprotein $E^{-/-}$ mice exposed to $PM_{2.5}$ showed increased content of lipids, cholesterol, and macrophages in atherosclerotic plaques, along with a reduced response to arterial dilatation. Also apolipoprotein $E^{-/-}$ mice fed an enriched fat diet exposed to PM presented an increased development of atherosclerotic plaques in comparison to animals receiving a balanced diet [17]. Finally, PM causes oxidative stress in cultured cells and cell-free systems [18–21].

The central objective of our research was to explore in vitro the capacity of PM to oxidize low-density lipoprotein (LDL) and to study the possible consequences of PM-induced LDL oxidation in the binding/internalization of PM-oxidized LDL (PM-ox-LDL) through the scavenger receptor (SR). SRs are involved with the development of foam cells by inducing internalization and accumulation of a large amount of ox-LDL within macrophages [22]. Since PM is internalized by macrophages through SR [23], the same receptor involved in ox-LDL uptake, we performed several assays to explore PM-ox-LDL and PM competition for the same receptor. Finally, to evaluate the effect of PM on a different group of receptors, we explored PM and native-LDL (nat-LDL) interaction with the LDL receptor (LDL-R).

MATERIAL AND METHODS

PM Sampling

$PM_{2.5}$ and PM_{10} were collected in the northern (industrial) zone of Mexico City, using high-volume samplers (1.13 m^3/min) (GMW model 1200, VCF HVPM10; Sierra Andersen, Smyrna, GA) on nitrocellulose membranes (Sartorius, Goettingen, Germany). Integrated 24-h samples were collected 5 days a week. PM was recovered from the membranes and handled as previously reported [24]. The chemical characterization of samples from the industrial zone of Mexico City has been reported elsewhere [2, 25].

Low-Density Lipoproteins

Human LDL (density = 1.019-1.063 g/mL) were isolated from the plasma of three randomly selected healthy blood bank donors who were in a fasting state by sequential density ultracentrifugation in potassium bromide at 4°C as previously described [26]. The experiments were performed separately on the LDL from each of the three donors. Lipoproteins were dialyzed first against 0.15 M NaCl and 0.01% ethylene diamine tetraacetic acid (EDTA) and then against phosphate buffered saline (PBS) to eliminate EDTA. LDL concentration is given in terms of its protein content determined by the bicinchoninic acid method (Pierce, Rockford, IL).

LDL Oxidation by $PM_{2.5}$ and PM_{10}

Isolated LDL were incubated with increasing doses of $PM_{2.5}$ or PM_{10} (20, 40, and 80 μg/mg LDL) with and without H_2O_2 (1 mM) over 24 h at 37°C, to evaluate oxidation by the thiobarbituric acid method (TBA) using a commercially available kit and following the manufacturer instructions (Northwest, Vancouver, WA, Canada). This method is based on the reaction of malondialdehyde with TBA, forming a complex that absorbs at 532 nm.

The PM dose range used in this study corresponds to concentration commonly reported in the literature as having significant in vitro cellular effects. They are also in a range relevant in magnitude to the exposure of an average person living in Mexico City at hot spots on airway bifurcations [27].

LDL Acetylation

Acetylated-LDL (ac-LDL) was prepared by LDL treatment with acetic anhydride and sodium acetate [28]. Briefly, an equal volume of saturated solution of sodium acetate was added to LDL with continuous stirring at a temperature of 4°C. Then, adequate amount of acetic anhydride was added to have a total mass of acetic anhydride equal to 1.5 times the mass of protein used and incubated for 1 h. Ac-LDL were dialyzed

against 0.15 M NaCl, centrifuged at 10,000 revolutions per minute (rpm) for 30 min and filtered.

LDL Fluorescent Labeling

LDL were labeled fluorescently with 1,1´-dioctadecyl-3,3,3´,3´-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes, Eugene, OR) as previously described [29]. LDL–DiI was dialyzed against 0.15 M NaCl and 0.01% EDTA as well as PBS to eliminate EDTA.

Cell Culture

We used Chinese hamster ovary (CHO), SR-transfected CHO (CHO-SR), and RAW264.7 cells. CHO and RAW cells were obtained from the American Type Culture Collection. CHO cells were used as a control due to the lack of scavenger receptor A (SR-A). CHO-SR were stably transfected with bovine SR-A (cells were kindly donated by Dr. Robert E. Pitas, Gladstone Institute of Cardiovascular Disease, University of California, San Francisco, CA). Cells were cultured in 10% fetal bovine serum–DMEM (Dulbecco´s modified Eagle´s Media)/F12 containing penicillin (50 U/mL)/streptomycin (50 μg/mL) for CHO, or geneticin (400 μg/mL) for CHO-SR. RAW cells (murine monocyte/macrophage) were used to compare SR activity in a naturally expressing SR cell line. Cells were grown in DMEM supplemented with 10% fetal bovine serum and penicillin (50 U/mL)/streptomycin (50 μg/mL) at 37°C in a 5% CO_2/95% air atmosphere.

PM-ox-LDL–DiI Binding/Internalization Assays

LDL–DiI were incubated with 80 μg PM_{10}/mg for 24 h at 37°C to induce oxidation, centrifuged at 5,000 rpm for 5 min to eliminate PM, and quantified once again. Confluent cells were incubated for 1 h in serum-free medium, and then in the presence of PM-ox-LDL–DiI (2.5, 5, 10, 20, and 40 μg/mL) for 4 h at 4°C (binding assay) or 37°C (internalization assay). Parallel assays were performed in the presence of 10 times the amount of nat-LDL and H_2O_2-ox-LDL to measure nonspecific binding internalization. Cells were washed five times with PBS–bovine serum albumin (2 mg/mL), recovered and analyzed by flow cytometry (FACScalibur instrument, Becton-Dickinson, NJ). To obtain specific binding/internalization data, fluorescence intensity values in the presence of an excess of lipoproteins (nat-LDL and H_2O_2-ox-LDL) were subtracted from total fluorescence intensity values to present final results.

Experiments were carried out using LDL from three different donors, and results are expressed as relative units where basal cell fluorescence corresponds to one relative unit. Relative units were used to normalize for the fluorescence variability observed between LDL–DiI samples obtained from different donors. Fluorescence variability of samples varied from 30 to 70 fluorescence units in the binding assays and between 170 and 280 fluorescence units in internalization assays, when a dose of 40 μg/mL of PM-ox-LDL-DiI was used in CHO-SR cells.

PM/LDL Competition Assays

Confluent CHO-SR were exposed to 0, 20, 40, and 80 μg/cm^2 of $PM_{2.5}$ or PM_{10}, and to 20 μg/mL of PM-ox-LDL-DiI or nat-LDL-DiI for 4 h at 4 or 37°C. Parallel assays were performed using an excess (10 times the amount) of nat-LDL and H_2O_2-ox-LDL as indicated above. Cells were washed, and samples were analyzed by flow cytometry. RAW cells were only tested using 40 μg/cm^2 of PM_{10} and 20 μg/mL of PM-ox-LDL–DiI or nat-LDL–DiI.

Cell Viability

Cell viability was assessed in parallel under the same experimental conditions described above by trypan-blue exclusion using a Neubauer camera. Trypan blue was added to cell suspensions at a final concentration of 0.8 mg/mL and incubated for 1 min at room temperature. The differentiation among the viable and nonviable cells was done through blue cell coloration under optical microscopy.

PM/LDL Interactions

We also evaluated possible PM–LDL (nat-LDL and PM-ox-LDL) binding interactions under cell-free conditions, using the same PM and LDL concentrations and conditions described in the binding/internalization experiments, except for the absence of cells in the assays. Samples were centrifuged and analyzed by fluorometry. We found that only ∼10% of PM-ox-LDL–DiI or nat-LDL–DiI total fluorescence was retained by PM. This indicates that "unspecific" PM binding to LDL introduced an experimental error no larger than 10%.

Statistical Analysis

All experiments were done in triplicate, using plasma from three independent donors. Graphics were created to show the average of the three independent

experiments ± standard deviation (SD). Data were analyzed by one-way analysis of variance (ANOVA) for competition assays and by factorial ANOVA for ox-LDL–binding/internalization assays, the last to evaluate the effect of ox-LDL concentration, cell line and the interaction between both factors (ox-LDL concentration × cell line). When necessary, variables were transformed to homogenize the variances. Both ANOVA procedures were followed by multiple comparison tests (Scheffe test) and specifically for factorial ANOVA, orthogonal contrasts were performed to determine differences between cell lines according to the ox-LDL concentration. Statistical analysis was performed using SPSS version 13.0 for Windows. The results were considered significant when $p < 0.05$.

RESULTS

LDL exposure to three increasing concentrations of $PM_{2.5}$ and PM_{10} (0, 20, 40, and 80 μg/mg LDL) for 24 h in the absence of H_2O_2 resulted in a concentration-related LDL oxidation. Exposures in the presence of H_2O_2 resulted in a corresponding increased oxidation (Figure 1). All further experimentation used PM_{10} + H_2O_2 induced LDL oxidation, due to limited accessibility to $PM_{2.5}$ samples.

Cell viability evaluation in cells (CHO, CHO-SR, and RAW) exposed to PM-ox-LDL and/or PM using the same experimental conditions used for binding/internalization and competition assays never resulted in viability decrements larger than 10% (data not shown). Results obtained from each experimental condition were adjusted according to viability.

PM-ox-LDL–binding/internalization assays in CHO cells showed a relatively low dose-related basal activity. In the case of CHO-SR cells, a higher dose-related PM-ox-LDL binding/internalization was observed ($p < 0.05$) (Figure 2).

Experimentation was also done using ac-LDL to investigate whether similar results would be obtained in our cell system when using traditionally modified LDL. The ac-LDL yielded similar results to the ones obtained with PM-ox-LDL using CHO-SR and CHO cells (data not shown).

PM-ox-LDL–binding/internalization competition assays in the presence of $PM_{2.5}$ or PM_{10} (20, 40, and 80 μg/cm^2) resulted in a PM concentration–related decreased binding (53%–55% at 80 μg/cm^2) (Figures 3A and 3C) and internalization (73%–78% at 80 μg/cm^2) ($p < 0.05$) (Figures 3B and 3D).

PM/LDL-R competition binding/internalization assays performed in CHO-SR cells showed that $PM_{2.5}$ and PM_{10} also interfered in a dose-related manner with nat-LDL binding (62% at 80 μg/cm^2) (Figure 4A) and internalization (86% at 80 μg/cm^2) ($p < 0.05$) (Figure 4B).

When RAW cells binding/internalization assays were performed, PM-ox-LDL binding was as low as observed in CHO cells, but internalization was as high as in CHO-SR cells ($p < 0.05$). Competition experiments showed that PM_{10} (40 μg/cm^2) induced decreased binding/internalization of PM-ox-LDL (22% and 32%, respectively; $p < 0.05$).

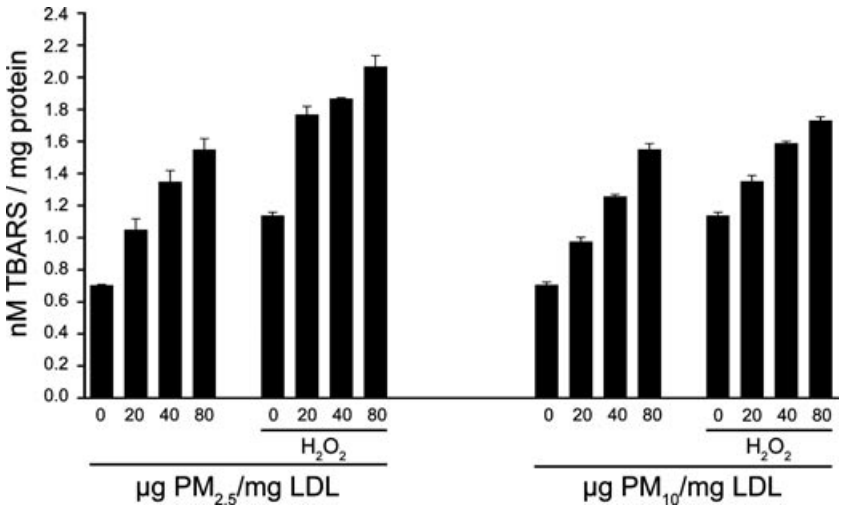

FIGURE 1. LDL oxidation by PM. LDL were exposed to increasing concentrations of $PM_{2.5}$ or PM_{10} (20, 40, and 80 μg/mg LDL) during 24 h at 37°C with and without H_2O_2, and LDL oxidation was observed. Thiobarbituric acid reactive substances (TBARS). Mean of three independent assays ± standard deviation (SD).

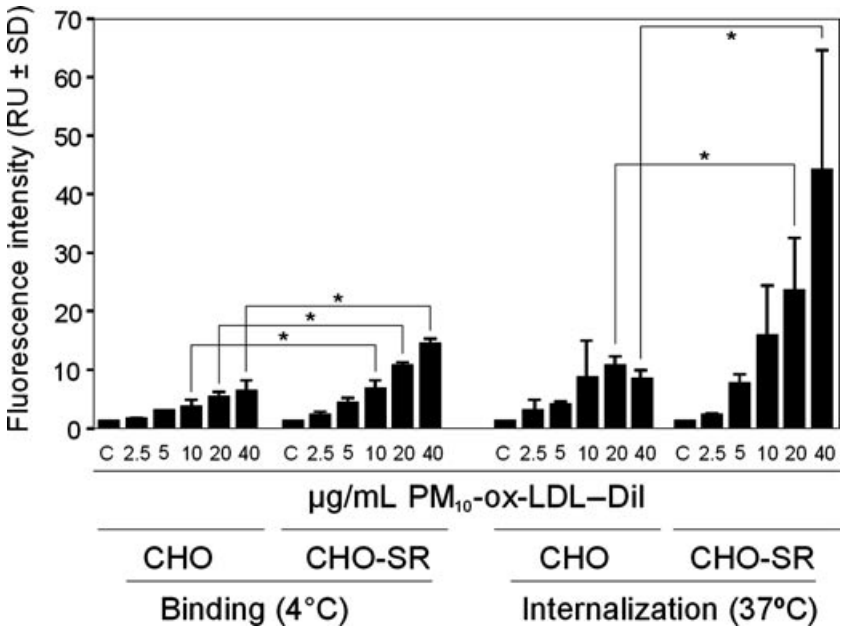

FIGURE 2. PM-ox-LDL binding/internalization by CHO and CHO-SR cells. CHO and CHO-SR cells were incubated with 2.5–40 µg/mL PM-ox-LDL-DiI for 4 h at 4°C (binding) or 37°C (internalization). LDL–DiI were previously oxidized with 80 µg PM_{10} for 24 h. Cells were recovered and evaluated by flow cytometry. Mean of three independent assays ± SD. * $p < 0.05$ vs. similar concentration in CHO cells.

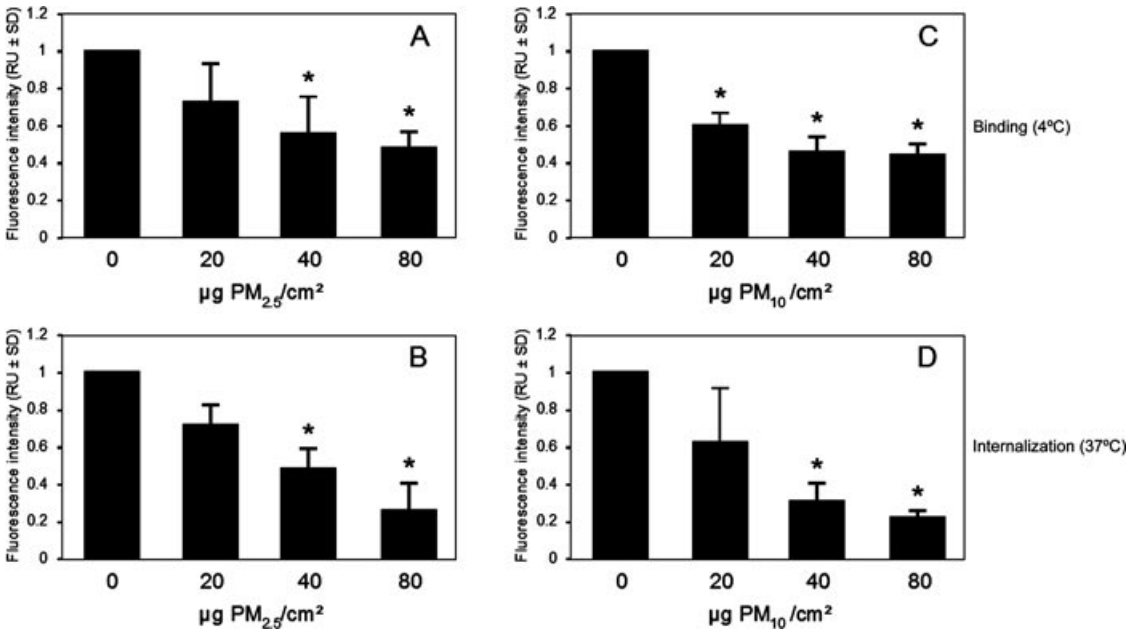

FIGURE 3. Competition of PM-ox-LDL and PM for SR-binding/internalization. Cultures of CHO-SR cells were exposed to 20, 40, and 80 µg/cm^2 of $PM_{2.5}$ (A, B) or PM_{10} (C, D), and 20 µg/mL PM-ox-LDL–DiI for 4 h at 4°C (A, C) or 37°C (B, D). Competition assays in the presence of $PM_{2.5}$ or PM_{10} resulted in decreased ox-LDL binding (53%–55%) and internalization (73%–78%) at 80 µg/cm^2. Mean ± SD of three independent experiments. * $p < 0.05$ vs. control.

Binding/internalization decrements were also observed in RAW cells when exposed to PM_{10} (40 µg/cm^2) and nat-LDL (45% and 55%, respectively; $p < 0.05$) (data not shown).

DISCUSSION

Evidence suggests that the mechanism by which PM exerts some of its adverse effects is through

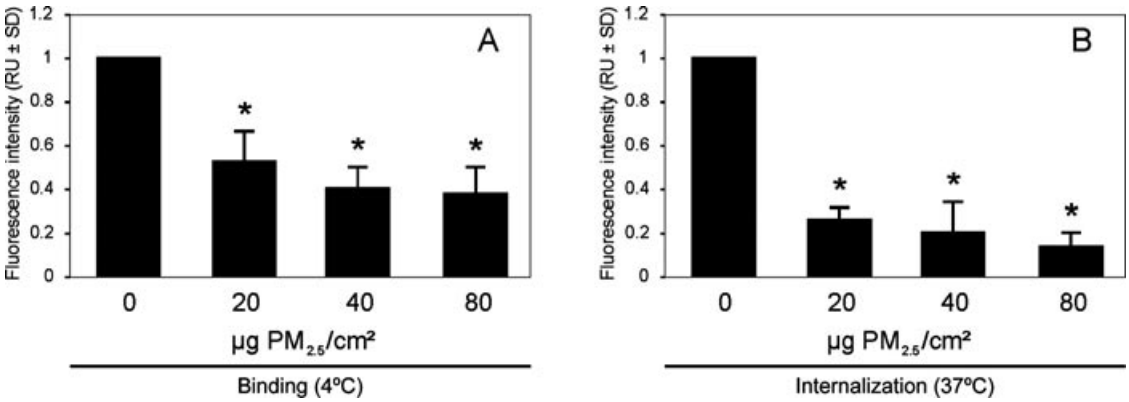

FIGURE 4. Competition of nat-LDL and PM for LDL-R binding/internalization. CHO-SR cells were exposed to 20, 40, and 80 μg/cm^2 of $PM_{2.5}$ and 20 μg/mL of native-LDL–DiI for 4 h at 4°C (A) or 37°C (B). PM interfered with nat-LDL binding (62%) and internalization (86%) at 80 μg/cm^2 with CHO-SR cells. Mean of three independent experiments ± SD. * $p < 0.05$ vs. control.

oxidative potential [18–21]. Several studies support the existence of a relation between atherosclerosis and exposure to PM in both humans [13, 14] and animals [15–17], without fully understanding of the mechanisms involved. Results presented in this paper support that PM is capable of oxidizing LDL. Both PM fractions, PM_{10} and $PM_{2.5}$, showed similar LDL-oxidative potentials in spite of differences in chemical composition [2]. PM-induced LDL oxidation seems to be mediated by metallic and nonmetallic PM constituents, since oxidation was induced in the presence/absence of hydrogen peroxide. Therefore, our experimental data support the hypothesis that PM could have the potential to aggravate atherosclerosis by promoting the oxidation of LDL and subsequent uptake by macrophages.

We report that PM is capable of competing for SR and the LDL-receptor blocking natural ligands binding. Although it is known that various types of particles (e.g., diesel, PM1648) [30–33] bind to SR, no one has previously reported that PM also alters LDL receptor functionality. This is an indication that PM could affect cell receptors with potential detrimental effects on cell function; the most obvious being the endocytic capacity of macrophages and a consequent altered primary immune response [34, 35]. Since SR is also present in other cell types like endothelial cells [36], it would be interesting to explore PM interactions with other receptors in various cell types.

The exact nature of PM/SR interactions and the mechanisms involved in the observed changes in receptor functionality remains to be described. These interactions may be related to receptor downregulation [37], direct physical PM/receptor interaction, or secondary to PM-related oxidative potential, as described under different conditions [38–41].

Since in this report we only studied PM-ox-LDL internalization through the type A scavenger receptor, studies evaluating the participation of other receptors involved in the atherosclerotic process, such as macrophage receptor with collagenous structure (MARCO), type B scavenger receptor, or lectin-type oxidized LDL receptor 1 (LOX-1), are still needed [42–44].

In spite of a growing body of research, mechanisms for PM-related cardiovascular effects are still not completely understood. For instance, we do not know whether PM can reach the bloodstream and directly affect vascular or cardiac cells, or if the effects observed in vivo are the result of inflammatory responses taking place in the respiratory system and then affecting the cardiovascular system by nervous or chemical mediators [45–50]. While it is possible that PM-induced systemic inflammation promotes the oxidation of LDL, our current study explored LDL oxidation in the event that PM or some of its compounds could enter the bloodstream and reach LDL.

In conclusion, we demonstrated that PM can oxidize LDL in a SR recognizable form. Although biological and epidemiological evidence relating PM exposure and atherosclerosis exists, the present work provides additional mechanistic evidence toward the elucidation of this phenomenon. We also observed that PM is able to induce changes in cell surface lipid receptors' functionality. This observation requires more studies since serious health implications would be related to these changes.

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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