British Journal of Medicine & Medical Research
17(7): 1-12, 2016, Article no.BJMMR.27433
ISSN: 2231-0614, NLM ID: 101570965

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Lipoprotein (a) Internalization by RAW 264.7 Cells is Associated with Morphological Changes and Accumulation of Lipids Detectable by Two-photon Scanning Microscopy

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Authors' contributions

This work was carried out in collaboration between all authors. Authors CS, AT, LT and EH contributed the original idea, coordination, methods, writing of the manuscript and data analysis. Authors MVP and AS provided protein preparation and analysis. Authors AT, EH and LT produced two-photon confocal microscopy images, while author PV produced confocal microscopy images. Author AS also provided revision of the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BJMMR/2016/27433
Editor(s):
(1) Shatrah Binti Othman, Department of Molecular Medicine, University of Malaya, Kuala Lumpur, Malaysia.
Reviewers:
(1) Rosa Maria Lino Neto Pereira, National Institute of Agrarian and Veterinarian Research, Portugal.
(2) Bradley Field Bale, Texas Tech University Health Sciences Center, USA.
(3) M. Rajajeyakumar, Dr. Mgr Medical University, Trichy, Tamilnadu, India.
(4) Anonymous, McMaster University, Ontario, Canada.
Complete Peer review History: http://www.sciencedomain.org/review-history/15943

Short Research Article

Received 31st May 2016
Accepted 8th August 2016
Published 26th August 2016

ABSTRACT

Lipoprotein (a) [Lp(a)] is an independent cardiovascular risk factor and its pathogenic mechanism is not completely clear. Lp(a) has been detected in atherosclerotic plaques and macrophages are one of the major cell types involved in atherogenesis. In order to characterize internalization of Lp(a) by RAW 264.7 cells, an established model of mouse macrophages, cells were treated with

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Lp(a) samples purified from plasma by affinity chromatography, and evaluated by western blotting. By 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, a tetrazolium salt, assay, Lp(a) was found to be non-cytotoxic for the cells at all the concentrations tested (0.0165-1.65 mg/ml). An ELISA performed on the lysate of Lp(a)-treated cells allowed to identify the highest intracellular accumulation of Lp(a) at 72 h treatment. Already at 24 h, however, important morphological alterations were detected upon Oil red and Nile red staining. A three-dimensional reconstruction obtained by two-photon scanning microscopy of the intracellular distribution of Nile red stained structures in treated cells shows preferential uptake of lipids in extra-nuclear regions. These data are useful to clarify the temporal and spacial aspects of intracellular accumulation of Lp(a) in RAW 264.7 cells and pose new bases for future studies on intracellular Lp(a) accumulation.

Keywords: Lipoprotein (a); macrophages; THP-1; internalization; two-photon microscopy; 3D reconstruction.

1. INTRODUCTION

Cardiovascular diseases are very common in Western industrialized countries, where they are the major cause of death. Beyond predisposing factors that cannot be modified (such as age, male sex, family predisposition), controllable factors include increased Low Density Lipoprotein (LDL) cholesterol and triglycerides, decreased High Density Lipoprotein (HDL) cholesterol, hypertension, type 2 diabetes mellitus, obesity and smoking. Lipoprotein(a) [Lp(a)], whose composition is similar to that of LDL in terms of cholesterol, triglycerides, phospholipids and apoB100, has also been identified as a putative genetic risk factor for atherosclerotic diseases because of its potential pro-atherogenic, prothrombotic and antifibrinolytic properties [1-5].

Serum levels of Lp(a) are classified into four classes, which correlate to increasing risk for cardiovascular disease. An Lp(a) level <20 mg/dl is considered desirable, whereas a level of 20 to 30 mg/dl is borderline, 31 to 50 mg/dl is high risk, and >50 mg/dl is very high risk. The unique and distinctive component of Lp(a) is the apolipoprotein(a) [apo(a)] glycoprotein, a member of the plasminogen gene family [6]. Apo(a) is disulfide linked to the apoB100 of the LDL-like particle. It is known to be a very heterogeneous glycoprotein that shares at least 80% homology with the plasmin precursor plasminogen and includes several types of kringle domains [7]. Among them, apo(a) kringle IV domains can be classified into 10 types (KIV_1-KIV_{10}) on the basis of amino acid sequence [8]. Kringle IV type 2 domain (KIV_2) is present in varying numbers of copies (from 3 to 48), which constitutes the basis of Lp(a) isoform size heterogeneity in humans [9-11]. The concentration of Lp(a) is largely genetically determined, inversely related to the number of KIV_2 repeats and not significantly modified by dietary or common pharmacological interventions.

The study by the PROCARDIS group, which has analyzed the genotype of 16.000 European subjects, showed that, among the different variants of the *LPA* gene, two in particular are associated with increased plasma levels of Lp(a) and are associated with a high risk of developing coronary heart disease and stroke. The majority of subjects that reported the *rs10455872* genetic variant have an isoform of Lp(a) characterized by a number of KIV_2 between 17 and 20, and in the presence of the *rs3798220* variant the isoform is characterized by a number of repeats comprised between 19 and 21. Both of these pathogenetic forms show a low molecular weight within the pool of Lp(a) isoforms recognized in the general population [3,12,13] and the epidemiological importance of these parameters is clear if we consider that 20% of the Western population has levels of Lp(a) above 50 mg/dl and approximately 40% of the white population has a number of KIV_2 lower than 22 [14].

Lp(a) is present in the arterial wall of atherosclerotic lesions and the extent of the accumulation in these sites is proportional to Lp(a) plasma concentration [15,16]. In fact, Lp(a) is accumulated like all LDLs and involves the recruitment of macrophages. These cells incorporate the lipid component and are transformed into foam cells which penetrate the intima-media and form fatty streaks. Here, macrophages also release cytokines and growth factors that lead to the proliferation and migration of vascular smooth muscle cells from the intima-media and contribute to plaques formation and progression.

The purpose of this project was to monitor the accumulation of Lp(a) in mouse RAW 264.7 cells, an established *in vitro* model of macrophages, exploiting both classical methods (ELISA, Oil red and Nile red staining) and two-photon scanning microscopy, an innovative technique which allows 3D reconstruction of the distribution of intracellular fluorescence in real time.

2. MATERIALS AND METHODS

2.1 Cell Line

The murine macrophage-like RAW 264.7 cell line (ATCC, Manassas, VA) was cultured in Dulbecco's modified Eagle's medium (DMEM, Lonza) supplemented with 10% FBS, 1% penicillin, 1% streptomycin and 1% L-glutamine and incubated at 37°C in 5% CO_2. Cells were routinely passed by scraping and re-seeding.

2.2 Lp(a) Purification

CNBr-activated Sepharose 4 (Amersham Biosciences) is a pre-activated medium for immobilization of ligands containing primary amines. This resin was coupled with a rabbit anti-human Lp(a) polyclonal antibody (DAKO) according to the manufacturer's protocol. In order to purify Lp(a), plasma from a healthy donor, kindly donated by the immunotransfusional service of the IRCCS San Matteo Foundation, was incubated with the anti-Lp(a) affinity matrix, previously equilibrated in phosphate buffered saline (PBS) in an end-over-end mixer at 4°C overnight. The gel was loaded onto a column and washed with PBS made up to 0.35 M NaCl, pH 7.0. Lp(a) was eluted with 100 mM glycine pH 3.0 and 2 ml fractions containing 200 µl of 1.0 M Tris pH 8.0 were collected, pooled, concentrated on an Amicon Ultra-15 Centrifugal concentrator (Millipore) and then dialysed by diafiltration against PBS on the same concentrator.

2.3 Protein Quantification

Lp(a) concentration of purified samples was determined with the Macra® Lp(a) Test Kit (Trinity Biotech), a sandwich ELISA which specifically detects the apo(a) moiety of Lp(a). The monoclonal antibody was used to coat the wells of a microtiter plate and used to capture Lp(a) from the sample during a one-hour incubation at room temperature. After washing, a polyclonal anti-Lp(a) horseradish peroxidase (HRP) conjugate was added and incubation performed at room temperature for 20 min. The plate was then washed and a chromogenic substrate for horseradish peroxidase (o-phenylenediamine) was provided to produce a coloured solution. After 20 min, the reaction was stopped with sulfuric acid and the concentration of Lp(a) (mg/dl) was quantitatively determined by comparison of the absorbance of the sample at 492 nm with a standard curve prepared with known concentrations of Lp(a).

2.4 Western Blot

Purified Lp(a) preparations were checked by Western blot analysis [17]. Lp(a) and molecular weight markers (Technoclone) were separated on a 1% agarose gel and transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA). The membrane was blocked with 5% (w/v) milk powder in PBS containing 0.1% Tween (PBS-T). For analysis of Lp(a) quality, the membrane was incubated with the rabbit polyclonal anti-human Lp(a) antibody (DAKO) at 1 µg/ml in 1% Bovine Serum Albumin (BSA) in PBS-T for 1 h. The blot was washed 3 times (5 min each) in PBS-T, incubated with an HRP-conjugated anti-rabbit antibody (GE Healthcare Europe GmbH, Milan, Italy) in PBS-T, 1% BSA for 1 h, washed 3 times as above, and visualised by ECL (GE Healthcare Europe GmbH, Milan, Italy).

2.5 Cell Viability Assays

For the viability tests, cells were inoculated in 96-well tissue culture plates at $25x10^3$ cells/well in 100 µl medium and incubated with increasing concentrations of purified Lp(a). Cell survival was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, a tetrazolium salt (MTT), assay [18]. Cells were incubated with 20 µl MTT at 37°C for 2 h, and then centrifuged at 1000 g for 10 min. The supernatants were then removed, the cells dissolved in 100 µl of dimethyl sulfoxide (DMSO), and the absorbance measured at 570 nm.

2.6 Lp(a) Uptake

RAW 264.7 macrophages were seeded in a 96-well plate at a density of $25x10^3$ cells/well and incubated with purified Lp(a). In order to analyse the intracellular accumulation of Lp(a), an ELISA method was set up. The cells were incubated with 100 µl of RIPA buffer (25 mM Tris HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) on ice for 15 min and the supernatants collected. An Immulon 96-well

plate (Santa Cruz Biotechnology, Inc) was pre-coated with polyclonal anti-human Lp(a) antibody (DAKO) at the final concentration of 20 µg/ml in coating buffer (50 mM sodium carbonate, pH 9.5) overnight. After washing with PBS-T, the wells were blocked with 200 µl of 2% (w/v) BSA in PBS-T for 1 h. Cell lysates were then added to the wells in 100 µl volumes at room temperature for 1 h. After washing, the wells were incubated with 100 µl of a purified anti-Lp(a) monoclonal antibody (2 µg/ml), whose hybridoma was kindly donated by Dr Livia Visai (Department of Molecular Medicine, University of Pavia, Italy), at room temperature for 1 h. The wells were washed and then incubated with 100 µl of HRP-rabbit anti-mouse IgG (1:500 dilution) at room temperature for 1 h. The colour reaction in each well was developed at room temperature using 100 µl of tetramethylbenzidine (TMB, Sigma) chromogenic solution, obtained by dissolving 1 mg TMB in 1 ml DMSO and then adding it to 9 ml of 0.05 M Phosphate-Citrate Buffer, pH 5.0 and 2 µl of fresh 30% hydrogen peroxide. The reaction was stopped with 100 µl of 2 M sulphuric acid. The absorbance was then read at 450 nm.

2.7 Microscopic Evaluation of Lipid Accumulation

RAW 264.7 cells were seeded at a density of 10^5 cells/glass slide and treated with Lp(a). The cells were fixed at room temperature with 4% paraformaldehyde in PBS for 10 min and then stained at room temperature with 0.3% Oil Red for 25 min, hematoxylin at room temperature for 3 min, rinsed in 60% isopropanol, and mounted with glycerin jelly for light microscopic evaluation. Alternatively, slides were stained with Nile Red and read at a Leica confocal TCS SP5 II microscope using an excitation wavelength of 543 nm, and an emission range of 580-670 nm.

2.8 Two-photon Scanning Microscopy

Multiphoton excitation fluorescence microscopy is a powerful technique for living tissues imaging. It is sometimes preferred to confocal microscopy due to its reduced phototoxicity, deeper tissue penetration, and reduced background noise detection [19]. Indeed, two-photon fluorescence does not require additional pinholes to remove out-of-focus signals and background noise. This technique preferentially requires femtosecond laser sources, necessary to easily excite nonlinear sample response. The two-photon scanning microscope used was developed at Femtolab, University of Pavia, assembled with a Ti:Sapph fs laser (Tsunami, Spectra Physics). Its main characteristics are an average output power up to 2 W at a repetition rate of 80 MHz and a pulsewidth of the order of 100 fs, in the wavelength tunability range extending from 750-990 nm. The sequence of frames was captured in video rate mode at 2 Hz, by means of a CCD camera. The scan mode was implemented with a line-scanning technique. The wavelength used was 830 nm, the average power from the laser 250 mW and the power impinging on sample 60 mW. The scanned field was 150x112 μm^2. Images were taken at different focus depths, each step being 0.100 µm. The emission filter was a short pass filter with a cut-off wavelength of 720 nm. The numerical aperture was 1.4 and magnification 60x.

2.9 Statistical Analysis

Kruskal-Wallis non–parametric test followed by Bonferroni-type *post-hoc* analysis was used for multiple comparisons.

3. RESULTS

3.1 Lp(a) Purification

Lp(a) was purified from the plasma of a homozygous donor. Homozygous donors have the advantage of presenting both alleles encoding the same number of KIV-2 repeats and therefore show a single apo(a) band in western blotting. The fractions corresponding to the affinity chromatography elution peak were concentrated and dialyzed against PBS. Lp(a) concentration was measured by Macra Lp(a) kit, with a final yield of 16 µg/10 ml plasma. This yield, though low, was suitable for subsequent experiments. Sample integrity was verified by Western Blot (Fig. 1), which also allowed to determine that the number of apo(a) KIV_2 repeats of the homozygote sample was comprised between 23 and 27.

3.2 Cytotoxicity

Lp(a) was tested at 0.0165, 0.165 and 1.65 mg/ml on RAW 264.7 cells for 24 h. All concentrations were found to be non-toxic for the cells (Fig. 2a, P=.126). Because of the observed tendency of Lp(a) to precipitate at concentrations higher than 1 mg/ml, 0.165 mg/ml was selected as the concentration to be used in the following experiments. Viability was determined for this treatment concentration at 8, 24, 48 and 72 h, the time-points selected to be used for the determination of Lp(a) internalization (Fig. 2b).

No significant cytotoxicity was observed versus control (P=.144).

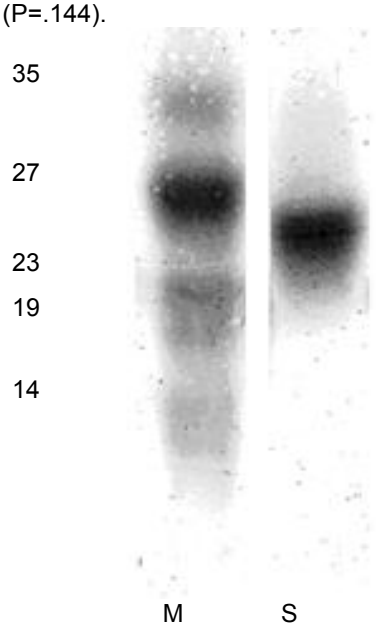

Fig. 1. Western blot analysis of purified Lp(a). M: Apo(a) Isoform standard (Technoclone). Numbers refer to the KIV_2 repeats of each isoform; S: sample. By comparison with the marker, it was possible to identify the number of KIV_2 repeats of the purified samples as comprised between 23 and 27

3.3 Lp(a) Uptake

The uptake of Lp(a) by RAW 264.7 cells was evaluated both at the molecular level, by immunodetection of apo(a) in the cell lysate, and at the cellular level, by staining cells seeded on slides. A significant level of intracellular apo(a) was detectable by ELISA only at 72 h incubation time (Fig. 3, P=.0025). At all other time-points, the treated samples showed values similar to control. Cells stained with Oil Red/Hematoxylin appeared pale in control conditions and much darker in treated samples (Fig. 4a and 4b, respectively). The staining had a diffuse aspect and only very few, isolated, small red spots were visible. Also, control cells typically preserved a round or oblong morphology throughout the experiment (Fig. 4a). In contrast, starting at 24 h incubation time, treated cells acquired an irregular shape and a flat aspect. This difference in morphology can be observed in much greater detail in classical confocal microscopy images (Figs. 5a and 5c) and in two-photon confocal microscopy images (Figs. 6a and 6c), which allow also to appreciate the roughness of the cell surface in the treated sample. Upon treatment with Lp(a), thin, pale filaments became visible departing from the cell body, thus that cells had a star-like shape (Fig. 4b). When stained with Nile Red (Fig. 5), Lp(a) treated cells showed a brilliant diffuse red staining (Fig. 5d) at 24 h, a typical feature induced by neutral lipids deposition like triglycerides and cholesterol [20], which were not at all visible in control cells (Fig. 5b). At longer incubation times, the intensity of the red staining became similar in control and treated cells.

3.4 3D Reconstruction of Intracellular Nile Red Fluorescence by Two-photon Scanning Microscopy

In order to distinguish outer membrane fluorescence from a true intracellular distribution of lipids, a 3D reconstruction of Nile Red fluorescence in cells exposed to 0.165 mg/ml Lp(a) for 24 h was performed by two-photon scanning microscopy. Fig. 6 shows control (a) and treated (c) cells in transmitted light and in fluorescence. The control, as for the classical confocal images, does not show any fluorescence (Fig. 6b). A bright fluorescence is instead detected in treated cells (Fig. 6d).

Of this field, a 0.1 μm slicing was performed. The single frames are shown in Fig. 7a (in grey) and Fig. 7b (in red and green, where green represents higher density points) and rotational videos are proposed in the attached files (Supplementary materials). A single cell, highlighted in a white rectangle in Fig. 6c and 6d, was also cropped (Fig. 6e and 6f) and a video built with all the 40 available frames (also attached). Fig. 6g and 6h show the same image of 6d in false colours. In Fig. 6g, the regions with higher fluorescence intensity are highlighted in green, while in Fig. 6h the boundaries of the detected fluorescence are well visible.

All the treated cells analysed showed a diffused distribution of fluorescence intensity. No droplets were observed. Lipid accumulations were also detectable as higher density regions, typically located in the central part of the cells. This finding is consistent with all the other microscopic observations here reported, which located the internalized lipids as a diffused distribution throughout the cell volume.

4. DISCUSSION

In order to study internalization of Lp(a), a novel cardiovascular risk factor not yet well characterized, by macrophages, RAW 264.7

cells were chosen. They are an immortalised mouse macrophage cell line which maintains a monocyte/macrophage morphology with a high pinocytose and phagocytose activity. Compared with human suspension THP-1 cells, they have the great advantage of growing as adherent cells without the need of specific stimuli.

Lp(a) was purified from the plasma of a homozygote donor and, according to the number of KIV_2 repeats (comprised between 23 and 27), it can be considered a representative of an intermediate cardiovascular risk group. The method applied here for isolation (affinity chromatography) allows a single-step separation of Lp(a) according to its capacity to bind to anti-Lp(a) antibodies, making it highly selective and providing a highly pure sample.

Regarding the cellular lipid staining protocols adopted, it is relevant to notice that both Oil Red and Nile Red are capable of a greater solubility in lipids than in water and are therefore well suited to identify lipids. While the former can be observed in direct light, the latter is almost non-fluorescent in water and other polar solvents, but it becomes fluorescent in non-polar environments. In biology, Nile red can be used as a membrane dye, which can be readily visualised using an epifluorescence microscope, but it is also able to stain intracellular lipid droplets which can be detected by fluorescence microscopy and flow cytometry [21]. Exploiting this capacity, we did not observe a greater lipid droplet content of treated cells compared to control cells, but a diffused distribution of the staining which was completely absent in control cells up to 24 h and which corresponded to the whole cell morphology. We also performed a 3D reconstruction of cellular fluorescence of treated cells, which confirms the absence of discrete droplets and the presence of signal in all the

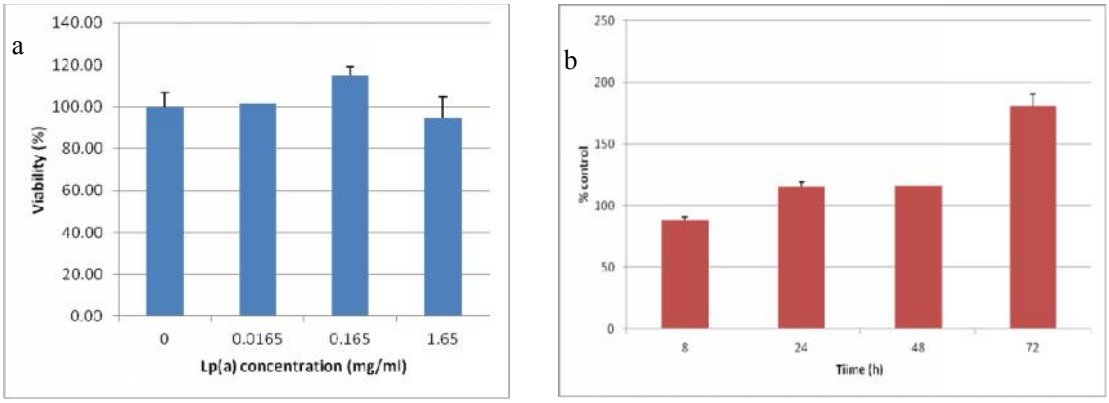

Fig. 2. Viability of RAW 264.7 cells (average ± SD), exposed to different concentration of Lp(a) (panel a), and to 0.165 mg/ml Lp(a) at different times (panel b), expressed as a percentage of control (n≥2, P=.126 and P=.144, respectively).

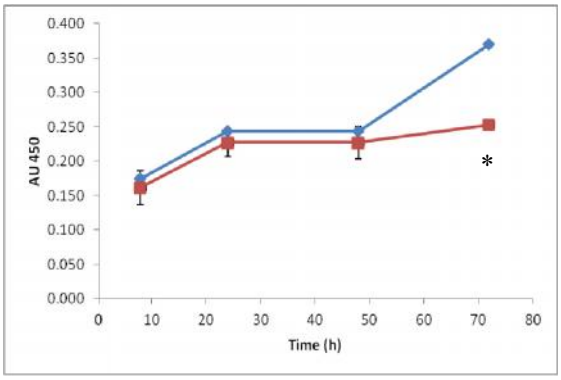

Fig. 3. Lp(a) internalization measured by ELISA (n=2) at different time-points. Data represent averages±SD of absorbance at 450 nm as a function of time. Blue line: treated sample. Red line: control. The asterisk indicates a statistically significant difference (P=.0025)

0.1 μm sections analysed. A higher fluorescence intensity is detected in the central part of the slicing, as also highlighted by false colour images.

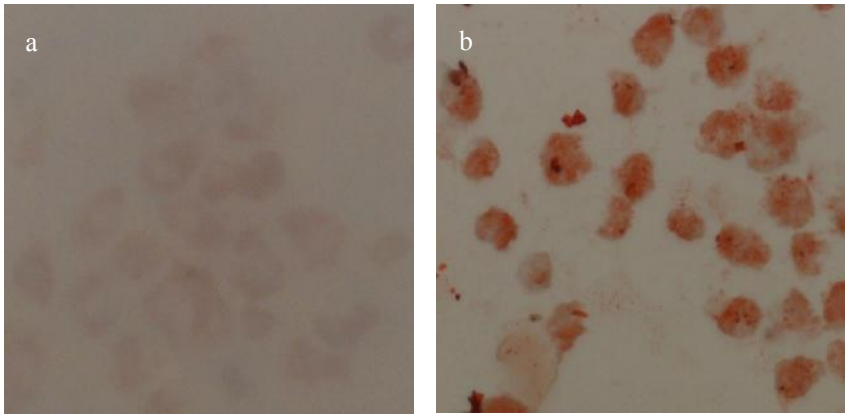

Fig. 4. Microscopic evaluation of RAW 267.4 cells after staining with Red Oil/hematoxylin. Representative images of (a) control cells and (b) Lp(a)-treated cells at 24 h incubation. Magnification: 40x

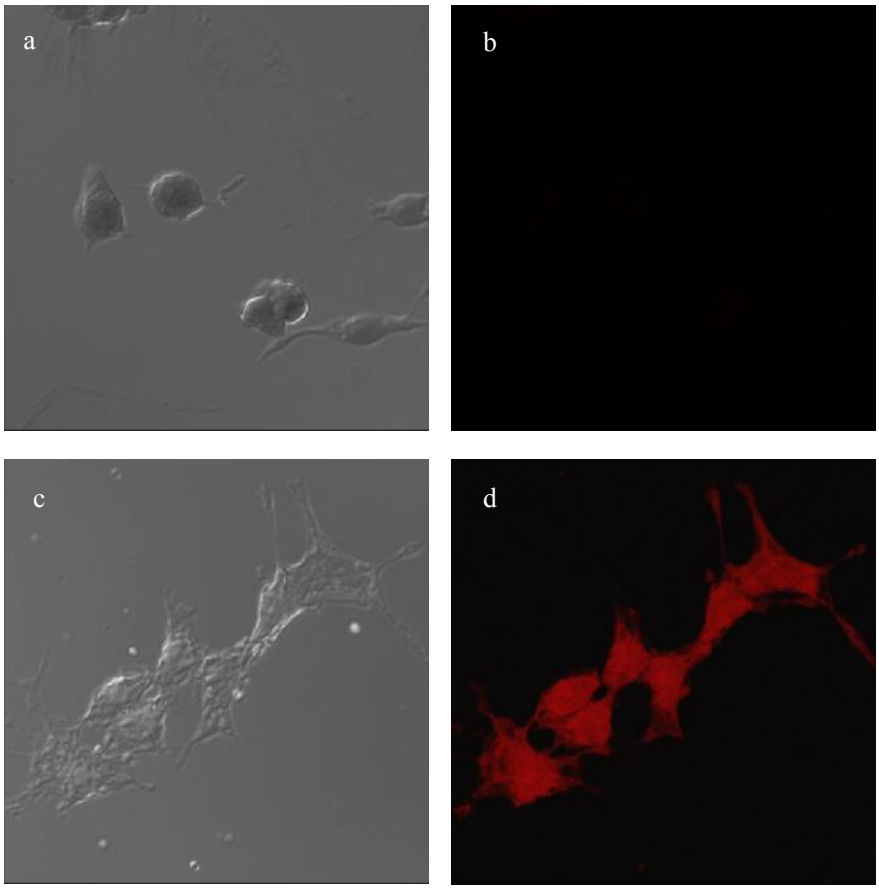

Fig. 5. Microscopic evaluation of RAW 267.4 cells after staining with Nile red. Images were taken under oil, at a 40x magnification, with a 3.5x electronic zoom, and a 1.3 optical aperture. (a, c) transmitted light ; (b, d) fluorescent light images of control (a, b) and treated (c, d) cells. Treated cells were incubated with 0.165 mg/ml Lp(a) for 24 h

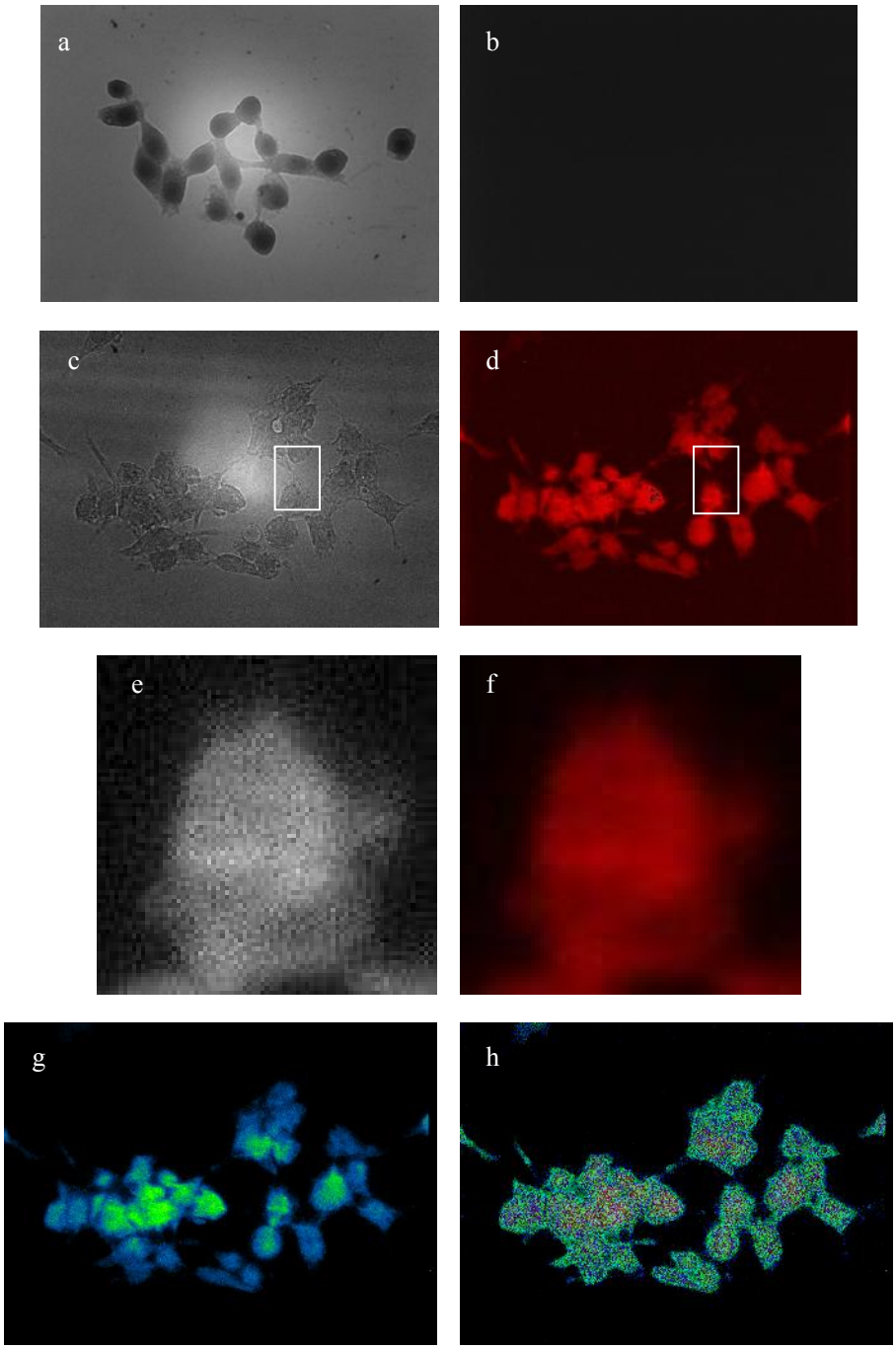

Fig. 6. Frames of the attached movies from a 3D reconstruction of Nile red stained cells observed by two-photon scanning microscopy. (a, c) Transmitted light of control (a) and treated (c) cells. (b, d) Fluorescent light images of control (b) and treated (d) cells. (e, f) Magnified rectangle highlighted in white in panels c and d. (e) Image of treated cells in false colours, where green indicates higher fluorescent intensity. (f) Image of treated cells in false colours, showing the boundaries of fluorescence. Treated cells were incubated with 0.165 mg/ml Lp(a) for 24 h. Panel (g) shows green fire and blue colouring of the image in (a), which underlines high density accumulation (firegreen zones) versus low density accumulation of fluorescence. (h) shows a 3-3-2-RGB version of the same image. Magnification: 60x.

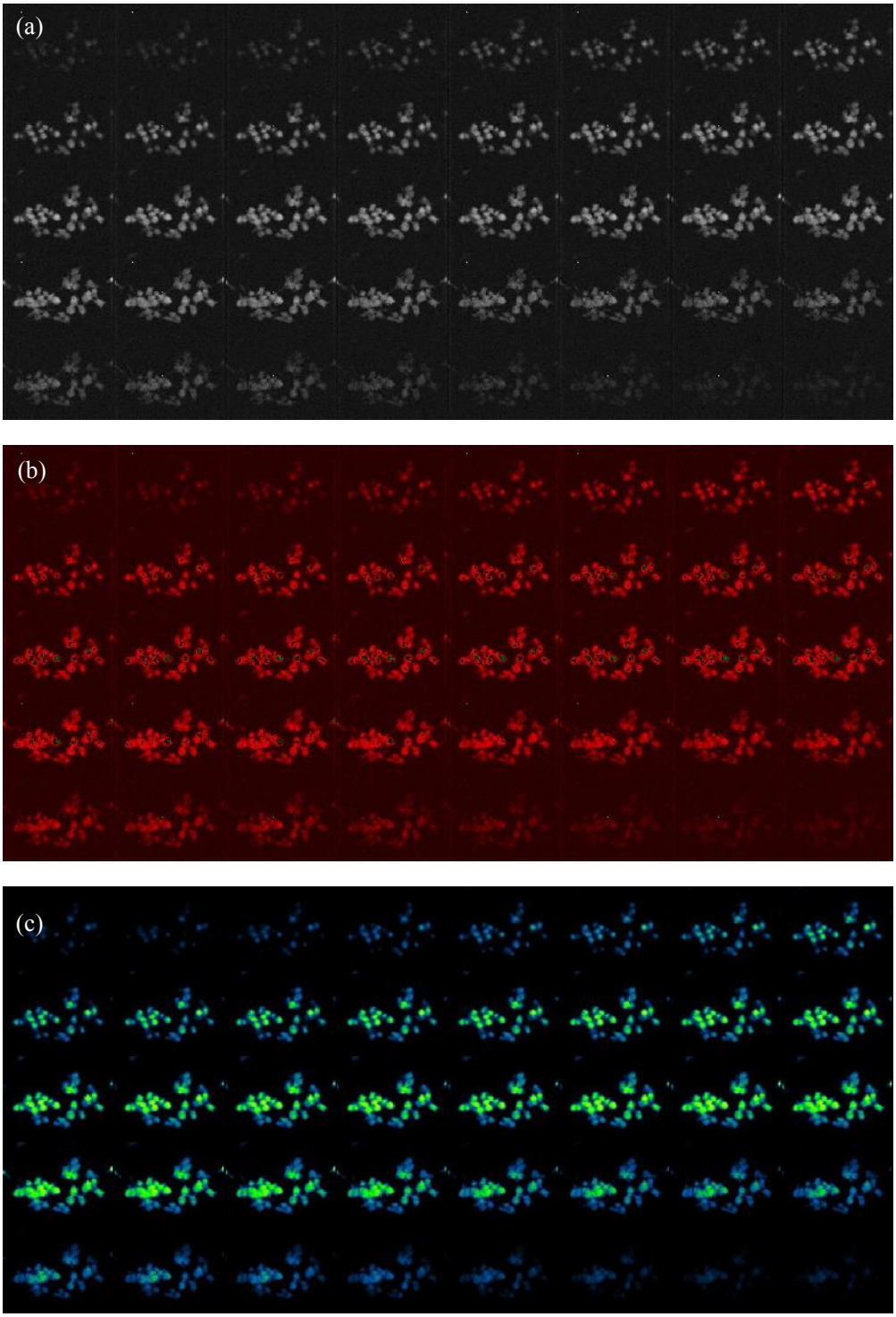

Fig. 7. The 40 frames of the attached video from a 3D slicing of Nile red stained cells observed by two-photon scanning microscopy (interval between frames: 0.1 μm). Cells were incubated with 0.165 mg/ml Lp(a) for 24 h. Panel (a) shows grey images, panel (b) red and green images. Green points represent higher density fluorescence points. See also Fig. 6g. Panel (c) shows green fire and blue images

According to the techniques we applied for Lp(a) uptake measurements, it was possible to recognise an event progression where the earliest cellular effect, at 24 h, is represented by a morphological change accompanied by lipid accumulation. Only later, at 72 h, an increased intracellular level of apo(a) was detected, suggesting a dissociation between lipid processing, which seems to occur faster, and apo(a) processing, which seems to occur more slowly. We noticed a significant background signal in untreated cells, which could depend on endogenous material cross-reacting with the anti-apo(a) antibody. However, the signal at 72 h was very strong and clear.

In humans, resident macrophages, which constitute the main class of cells infiltrating the atherosclerotic lesions, can internalize apoB lipoproteins (e.g. Lp(a)) through different mechanisms, including receptor-mediated endocytosis, phagocytosis and pinocytosis and degrade them in lysosomes, generating lipid droplets, typical of foam cells [22]. Unique receptors for Lp(a) have not been identified yet, but the binding can occur through other recognized receptors that are present in macrophages, such as VLDLR and Plg receptors. However, since Lp(a) is not present in mice, it is possible that its uptake does not occur following the same processes. The diffuse distribution of lipid staining that we observed is, indeed, in contrast with the droplet-like distribution typically found in human cells upon Lp(a) uptake [23]. This divergence of results could be due to different mechanisms carried out by murine macrophages during internalization of Lp(a) particles.

RAW 264.7 cells are known to differentiate into M1/M2 phenotypes upon several stimuli, something it might be worthwhile verifying in future experiments. In fact, we have already explored by Real Time Cell Analysis the capacity of Lp(a) to induce alterations in cell adhesion [19], and, according to our observations, this technique seems perfectly suited for this cell line as well. Similarly, as apo(a) accumulation seems to have a role in atherogenesis, it would be interesting to analyse if this effect depends on the number of KIV_2 repeats.

The data obtained from this model cell line could be also complemented with studies comparing internalization of Lp(a) with that of other lipoproteins (e.g. LDLs). Furthermore, as several extracellular matrix components, especially proteoglycans, which are particularly enriched in the arterial intima of atherosclerotic lesions [24, 25], are crucial for the binding of lipoproteins at these sites, it would be interesting to explore the binding of Lp(a) to such extracellular molecules, as opposed to other lipoproteins. In fact, this aspect could easily differ depending on the lipoprotein components that are available for the interaction, especially considering the unique nature of apo(a). Taken together, evidence on both Lp(a) cellular internalization and binding to extracellular matrix might influence alternative therapies aimed at controlling the atherogenic potential of Lp(a).

5. CONCLUSIONS

In summary, with this work we have demonstrated that RAW 264.7 cells are sensitive to the action of purified Lp(a). The internalization of Lp(a) by these cells was studied from both the apo(a) and lipids point of view. The main effect consists of a morphological change with the appearance of cell filaments and of a diffuse staining, better visible upon Nile red staining. A 3D reconstruction of Nile red fluorescence by two-photon microscopy indicates that lipids tend to accumulate throughout the cell and especially in their middle part.

These data allowed to establish that an ELISA-based method is apt to quantify apo(a) internalization by RAW 264.7 cells. This cell line seems also good to investigate Lp(a) internalization by non-human cells, which seems to occur with different modalities from the human one. Moreover, the high resolution 3D reconstruction of internal lipid distribution by two-photon scanning microscopy provides a technique potentially useful to follow the effect of therapeutic agents.

CONSENT AND ETHICAL APPROVAL

The plasma samples were obtained from a donor affiliated to the Immunohaematology and Transfusional Service of IRCCS San Matteo Foundation, Pavia, who had signed an informed consent according to the existing relevant Italian regulations.

ACKNOWLEDGEMENT

We would like to acknowledge useful discussions with Prof. Vittorio Molina, and to thank Dr. Paola Isernia and Prof. Laura Salvaneschi for the kind

supply of plasma bags. Funding for this project derived from MISE-ICE project n. 166-2010, which includes a contribution by Neomed S.r.l. and by Rottapharm S.r.l. Neomed S.r.l. also contributed to support VP's fellowship.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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