British Journal of Medicine & Medical Research
16(4): 1-12, 2016, Article no.BJMMR.26869
ISSN: 2231-0614, NLM ID: 101570965

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Real Time Cell Analysis of Model Target Cell Lines Exposed to Purified Lipoprotein (a)

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Authors' contributions

This work was carried out in collaboration between both authors. Author CS provided initial idea, statistical analysis and resources to perform the experiments. Author AS performed the experiments and provided the data. Both authors contributed to experimental design. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BJMMR/2016/26869
Editor(s):
(1) Arun Kumar Nalla, College of Medicine, University of Illinois, Peoria, IL, USA.
Reviewers:
(1) Rodrigo Moore-Cararsco, Universidad de Talca, Chile.
(2) Shashwatee Bagchi, University of Maryland School of Medicine, USA.
(3) M. Rajajeyakumar, Dr. Mgr Medical University, Tamilnadu, India.
Complete Peer review History: http://sciencedomain.org/review-history/14951

Original Research Article

Received 6th May 2016
Accepted 3rd June 2016
Published 8th June 2016

ABSTRACT

Lipoprotein (a) [Lp(a)] is a novel independent cardiovascular risk factor and it includes, beyond apoB100, apolipoprotein (a), whose molecular weight is dependent on the number of genetically encoded kringle IV type 2 repeats and inversely related with Lp(a) plasma concentration. Risk thresholds for molecular weights have been proposed, but there is not a full consensus and the role of the different isoforms in pathogenesis has not yet been clarified. The aim of the present work is to explore the biological effect of low and high molecular weight Lp(a) isoforms on cultured cells. Real-time impedance analysis has been performed on model cell lines of atherogenesis and Lp(a) metabolism (THP-1, HUVEC, HASMC and HepG2) using affinity purified Lp(a) with 22 (low number) and 31 (high number) kringle IV type 2 repeats, respectively. Normalized Cell Index data show that all the cell lines tested are modified by Lp(a), though with a variable intensity. Low and high molecular weight Lp(a) isoforms at similar concentrations can exert opposite modifications on

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the impedance kinetics of different cell lines. These data suggest that purified Lp(a) can modify the behaviour of adherent cell lines, an effect which can be detected as impedance variation and which is influenced by its specific isoform.

Keywords: Lipoprotein (a); kringle IV type 2; isoforms; HepG2; THP-1; HUVEC; HASMC.

1. INTRODUCTION

The presence of lipoprotein(a) [Lp(a)] in plasma was first described by Kare Berg in 1963, who identified it as an LDL-like particle [1]. Lp(a) has been recognised as a putative risk factor for atherosclerotic diseases [2,3] because of its pro-atherogenic, prothrombotic and antifibrinolytic properties. Levels of Lp(a) are classified into classes, which correlate with an increasing risk of cardiovascular disease (CVD) [4]. Lp(a) composition is similar to that of LDL in terms of cholesterol, triglycerides, phospholipids, and apoB100. The unique and distinctive component of Lp(a) is the apolipoprotein(a) [apo(a)] glycoprotein, a member of the plasminogen gene family, with a strong structural homology to plasminogen [5]. Apo(a), which is disulfide linked to the apoB100 of the LDL-like particle, is known to be a very heterogeneous glycoprotein including domains referred to as kringle IV, kringle V, and the inactive protease domain [6]. The apo(a) kringle IV domains can be classified into 10 types (KIV_1-KIV_{10}) on the basis of amino acid sequence [7]. Kringle IV type 2 (KIV-2) is present in a largely variable number of copies (from 3 to 48), which generates Lp(a) isoform heterogeneity in humans [8-10]. Low MW species are related to high plasma concentration and vice versa [11]. Different epidemiological studies have also suggested that Lp(a) could increase the risk of cardiovascular disease and ischemic stroke independently from plasma concentration if associated with other predisposing factors such as hypercholesterolemia, hypertension, diabetes mellitus and low level of HDL. Lp(a) is present in the arterial wall of atherosclerotic lesions: the extent of its accumulation in these sites is proportional to its plasma concentration [12,13] and involves recruitment of macrophages. Despite all these data, the physiological role of Lp(a) remains elusive, as the biology of its different isoforms. With this work, we wanted to exploit impedance kinetics, which is a global index dependent on cell morphology, adhesion and proliferation, to test if purified Lp(a) is able to alter the behaviour of key model cell lines and, if yes, if this effect is dependent on the concentration and/or on the MW of apo(a).

2. MATERIALS AND METHODS

2.1 Lp(a) Purification

Lp(a) was purified from plasma bags of homozygous healthy donors, carrying isoforms with 22 (K22, low MW) and 31 (K31, high MW) KIV-2 domain repeats, respectively, through affinity liquid chromatography. Western-blot positive fractions were pooled, sterilized by filtration (0.2 µm pore size filter, Millipore), concentrated, dialysed versus PBS buffer, using 30 KDa cut-off concentrators (Centricon, Millipore) and quantified using Macra® Lp(a) ELISA kit (Trinity Biotech).

2.2 Cell Lines

Human hepatocellular carcinoma cells (HepG2), Human Umbilical Vein Endothelial Cells (HUVECs) and human monocytic leukemia cells (THP-1) were obtained from ATCC; Human Aortic Smooth Muscle Cells (HASMCs) were purchased from Gibco® Life Technologies. All the cell lines were maintained in a standard 37℃ humidified incubator at 5% CO_2 and 10% foetal bovine serum in their respective culture medium. THP-1 cells were differentiated into macrophage-like cells by adding 5 ng/ml 12-O-tetra-decanoylphorbol-13-acetate (TPA, Sigma-Aldrich) 24 h before treatment.

2.3 Real Time Cell Analysis

Real Time Cell Analysis was performed monitoring impedance by using 8-wells E-Plates and iCELLigence (ACEA Biosciences), a system which allows label-free, real time impedance-based measurement of cell behaviour. The dimensionless parameter Cell Index (CI) represents the cell status and is directly proportional to number, proliferation, size, morphology and attachment of cells. The iCELLigence RTCA Station was kept in an incubator at 37℃ and 5% CO_2. For each cell line, density scouting showed that seeding 50000 cells/well was appropriate and treatments were performed in duplicate when cells were in their exponential phase. PBS was used as a control.

Impedance was monitored every hour before treatment and each minute for 2 h after treatment, then every 15 min for 122 h. Data analysis was performed using RTCA Data Analysis Software 1.0.0.1304 (ACEA Biosciences) and ANOVA followed by Tukey post-hoc test were calculated by OriginPro 8 v. 0.8724 (OriginLab corporation).

3. RESULTS

3.1 Lp(a) Purification

Elution profiles of Lp(a) isoforms typically produced a single peak, corresponding to homogeneous Lp(a). Typical yields of purified K22 and K31 Lp(a) were 39±4 µg and 17±3 µg per 10 ml plasma, respectively (n=4). Fig. 1 shows the result of a representative Western blot. The highest solubility of the isolated proteins in PBS was 80 µg/ml. For this reason, the highest concentration tested in the experiments here proposed is 20 µg/ml.

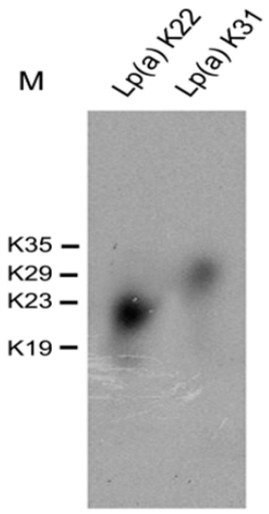

Fig. 1. Western blotting of purified K22 and K31 Lp(a) isoforms. Lane 1: K22 Lp(a) isoform; lane 2: K31 Lp(a) isoform

3.2 Real-Time Cell Analysis (RTCA)

Each cell line is characterised by a specific long-term cell impedance kinetic profile and typical Cell Index (CI) values, which represent the impedance fingerprint of a given cell line. In order to perform data analysis, the Normalized Cell Index (NCI) profiles were calculated by normalizing the Cell Index (CI) by its value at the last sampling time point before treatment.

3.2.1 HepG2 cells

Fig. 2a shows the Normalized Cell Index (NCI) profiles, calculated by normalizing the Cell Index by the time point immediately before treatment (23:51:51). It is possible to observe that, independently from isoform or concentration, the NCI profiles of HepG2 treated cells were systematically higher compared to control within 30 min after treatment (Table 1, row 1), indicating an early effect induced by Lp(a). All the NCI profiles then grew parallelly until they tended to converge on the control profile at ca. 7 h after treatment. The profiles of K31 at 10 and 20 µg/ml then diverged towards values lower than those of all the other samples (Table 1, row 2). Control cells and cells treated at low Lp(a) concentration (5 µg/ml) showed a peak in NCI between 40 and 74 h which was totally absent in cells treated with Lp(a) at 10 and 20 µg/ml, independently from the MW. In the long-term (74-120 h treatment), all the profiles tended to overlap.

Slope analysis in incremental 5 min intervals (Fig. 2b) revealed an inversion of sign for K22 at 10 and 20 µg/ml lasting for 10 and 30 min, respectively. This concentration-dependent effect was, in contrast, totally absent in the case of K31. A concentration-dependence of effect for both MWs was also detected when a dose-response curve (DRC) for the Area Under the Curve (AUC, Fig. 2c) was calculated for the initial 2 h of treatment (Square R: 0.98 and 0.81 for K22 and K31, respectively). K22 was therefore able to increase cell impedance compared to K31 within this interval, an effect which was clearer at higher concentrations (10 and 20 µg /ml) and consistent with the observation on slope.

3.2.2 THP-1 cells

All the NCI curves of THP-1 cells were characterized by a wavy pattern, which is the typical RTCA fingerprint of secreting cells. In these cells, Lp(a) induced a systematic increase of NCI with respect to control within 5 h after treatment, independently from isoform and concentration (Fig. 3a and Table 1, row 3). In general, the profiles of K22 samples showed a medium-term behaviour close to the control, while the K31 profiles tended to diverge towards higher NCI values (Table 1, row 4). Only at 84 h, K22 (20 µg/ml) started showing an aberrant behaviour, compared to all other samples, with a sudden increase of NCI beyond control values. An early, concentration-dependent effect was

observed in the initial 30 min treatment time, with K22 consistently showing a lower NCI compared to K31 (Fig. 3c). The different behaviour of K22 is also confirmed by the fact that its profile slope (Fig. 3b), calculated between 0 and 90 min treatment time, showed an important reduction at 20 µg/ml compared to K31 at the same concentration.

3.2.3 HUVEC cells

In the initial 6 h treatment (Fig. 4), all Lp(a) samples tested, except for K31 at 20 µg/ml, showed a mild, but consistent inhibitory effect on the NCI of HUVEC cells with respect to control (Table 1, row 5), followed by a strong superposition of profiles up to 64 h. Only after this long interval, a significant divergence was observed (Fig. 4a). To better analyse these complex profiles, the NCI values of control wells were subtracted from NCI values of each time point, generating Baseline Normalized Cell Index (BNCI) profiles (Fig. 4b). In the case of K31, a perfect dose-response correlation (Square R=1) was observed for maximum BNCI (Fig. 4c), while this relationship was absent in the case of K22. Looking at the average BNCI profiles, an early inhibitory effect was detectable within 6 h of treatment (30 h of analysis) and was consistent for all the treated wells, except for the highest concentration of K31. In the long term, all the samples reached an NCI value higher than control, a feature which was gained by K22 samples earlier than by the K31 ones (Fig. 4b and Table 1, row 6).

3.2.4 HASMC cells

HASMC cells show a peculiar behaviour compared to all the other cell lines, as they reach a CI peak at 8 h after seeding and start then to decline. For this reason, treatment was performed both at 24 h (Fig. 5a) and, for 10 µg/ml, also at 2 h (Fig. 5c). In both cases, it was possible to observe a complex behaviour. In the first case, overall, all the NCI profiles were similar in shape, except for a strong reduction in NCI induced in the initial 2 h by the highest concentration of K31 Lp(a) (20 µg/ml) tested versus all other curves (Table 1, row 7). In fact, a concentration-dependent effect on slope could be observed for K31, something absent for K22 (Fig. 5b). Within 16 h after treatment, both K22 Lp(a) and K31 Lp(a) isoforms resulted in early stimulation of proliferation/adhesion of HASMC cells compared to control (Table 1, row 8).

Table 1. Statistical analysis of curves in the given interval

Cell line	Row number	Sampling interval	F, df and P values	Type of significant comparison	Timepoints*
HepG2	1	24:13:50 – 24:40:50	F(6,56)=137.39 .000-.000	All treatments vs control	9
	2	40:12:51 – 44:42:51	F(6,56)=132.27 .000-.000	K31 (10, 20 µg/ml) vs control	9
THP-1	3	24:15:24 – 29:14:25	F(6,56)=331.24 .000-.020	All treatments vs control	10
	4	72:14:25 – 92:14:25	F(6,56)=90.05 .000-.000	K31 vs control	10
HUVEC	5	23.48.25-30:45:29	F(6,42)=10.35 .000-.052	Control and K31 (20 µg/ml) vs. each other treatment	7
	6	115:49:29-118:00:29	F(6,56)=3862.12 .000-.000	All treatments vs control	9
HASMC (24 h)	7	24:30:43-26:39:44	F(6,49)=20.25 .000-.000	K31 (20 µg /ml) vs. others	8
	8	28:24:44-30:24:44	F(6,49)=210.37 .000-.000	All treatments vs control	8
	9	60:09:44-62:24:44	F(6,56)=7370.87 .000-.000	K22 (20 µg/ml) vs. others	9
HASMC (2 h)	10	25:02:23-32:02:23	F(2,18)=878.10 .000-.000	K22 and K31 vs. control	7

** Number of time points used for ANOVA calculation by Origin8*

K22 at 20 µg/ml showed a persistent reduction of NCI compared to control and to all other samples starting at 60 h (Table 1, row 9). In the second RTCA experiment, performed treating HASMC cells with 10 µg/mL Lp(a) 2 h after seeding, the NCI profiles confirmed a stimulatory effect induced by both isoforms with respect to control (Fig. 5c and Table 1, row 10), persisting throughout all the time of analysis. Also BNCI curves of K22 and K31 were similar in pattern (data not shown) and K22 showed an earlier and stronger effect on cells compared to K31, especially evident at 30 min after treatment (Fig. 5d).

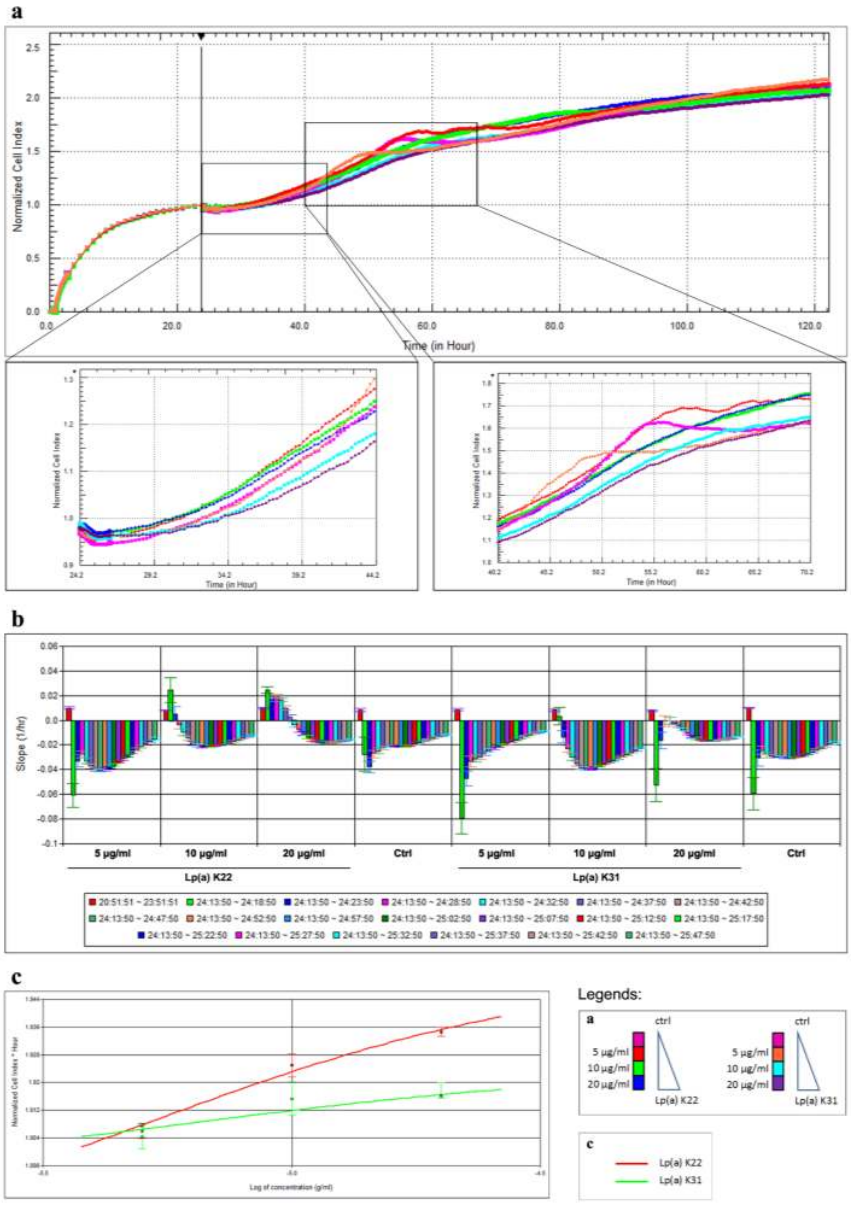

Fig. 2. Real Time Cell Analysis (RTCA) of HepG2 cells exposed to Lp(a) represented as Normalized Cell Index (NCI). Normalization was performed by dividing Cell Index (CI) by its value at the last time point before treatment. (a) Full profile with magnification boxes of two intervals (24.0-44.0 h and 40.2-70.2 h); (b) Histogram representation of slope values in incremental time intervals; (c) NCI versus logarithm of Lp(a) concentration

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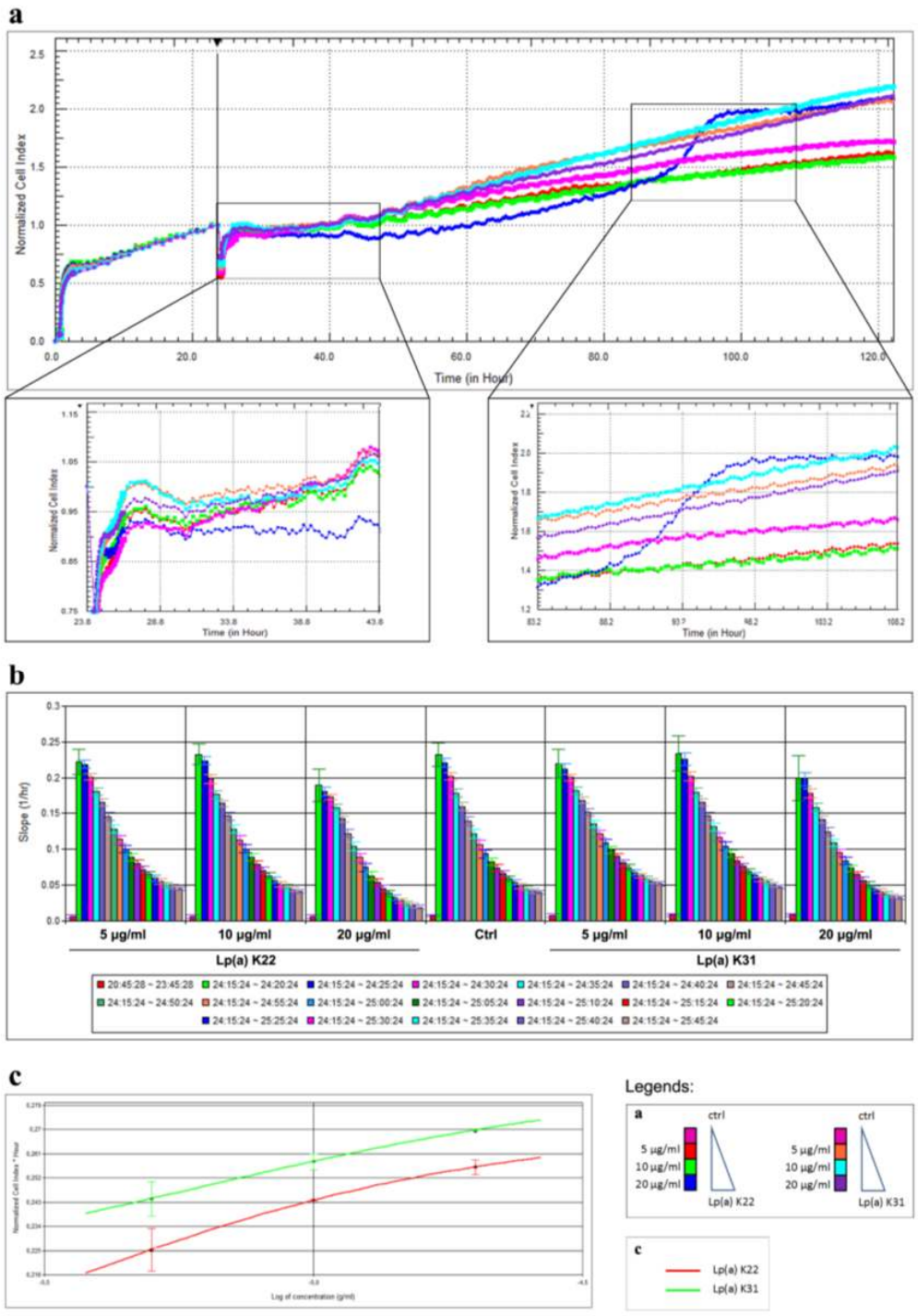

Fig. 3. Real Time Cell Analysis (RTCA) of THP-1 cells exposed to Lp(a) represented as Normalized Cell Index (NCI). Normalization was performed by dividing Cell Index (CI) by its value at the last time point before treatment. (a) Full profile with magnification boxes of two intervals (23.8-43.8 h and 83.2-108.2 h); (b) Histogram representation of slope values in incremental time intervals; (c) NCI versus logarithm of Lp(a) concentration

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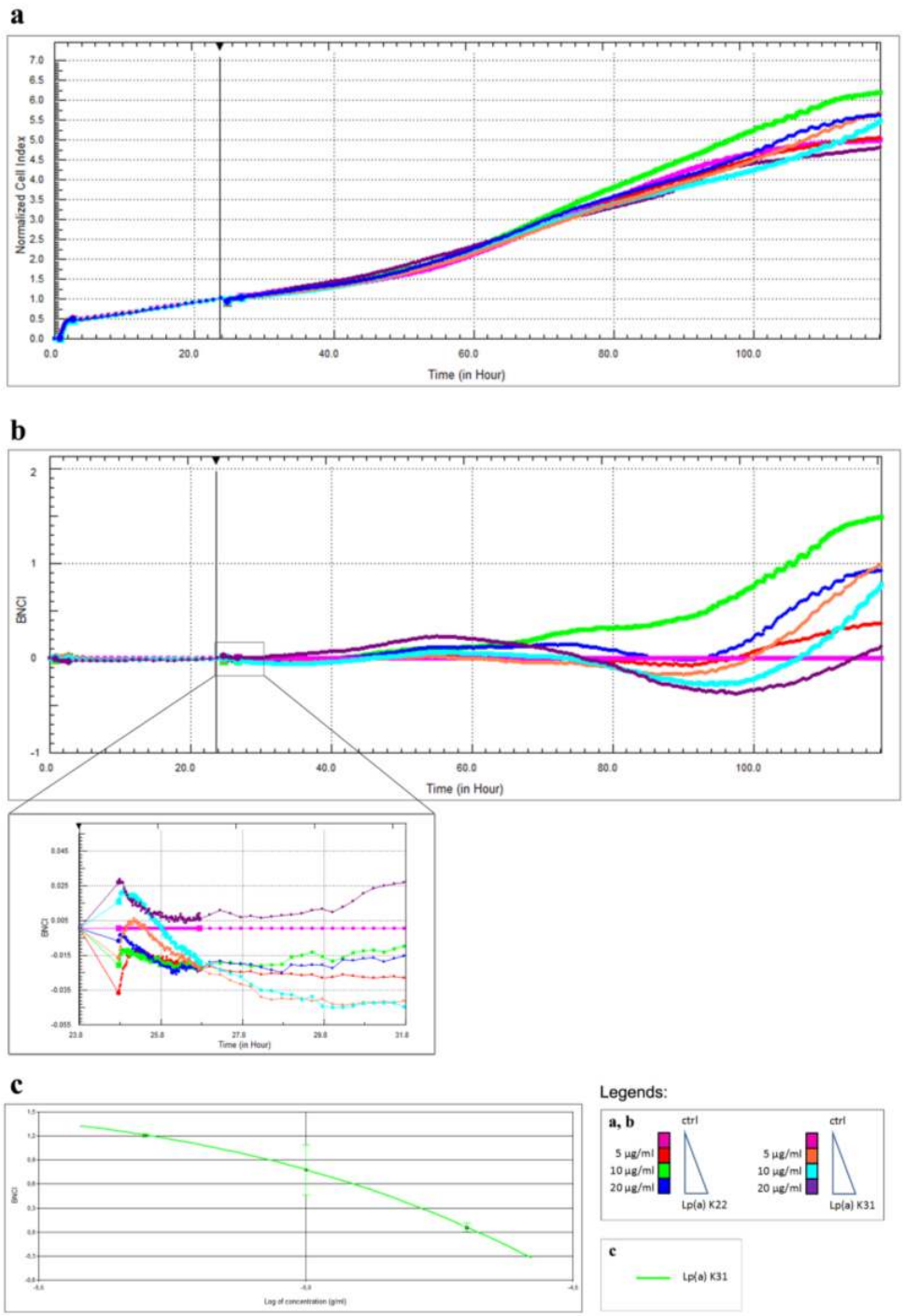

Fig. 4. Real Time Cell Analysis (RTCA) of HUVEC cells exposed to Lp(a) represented as Normalized Cell Index (NCI), (a), and Baseline Normalized Cell Index (BNCI), (b). Normalization was performed by dividing Cell Index (CI) by its value at the last time before treatment. BNCI was calculated by subtracting the control profile. In panel (b), a magnification box of the 23.8-31.8 h interval is shown; (c) BNCI versus logarithm of Lp(a) concentration for the K31 isoform.

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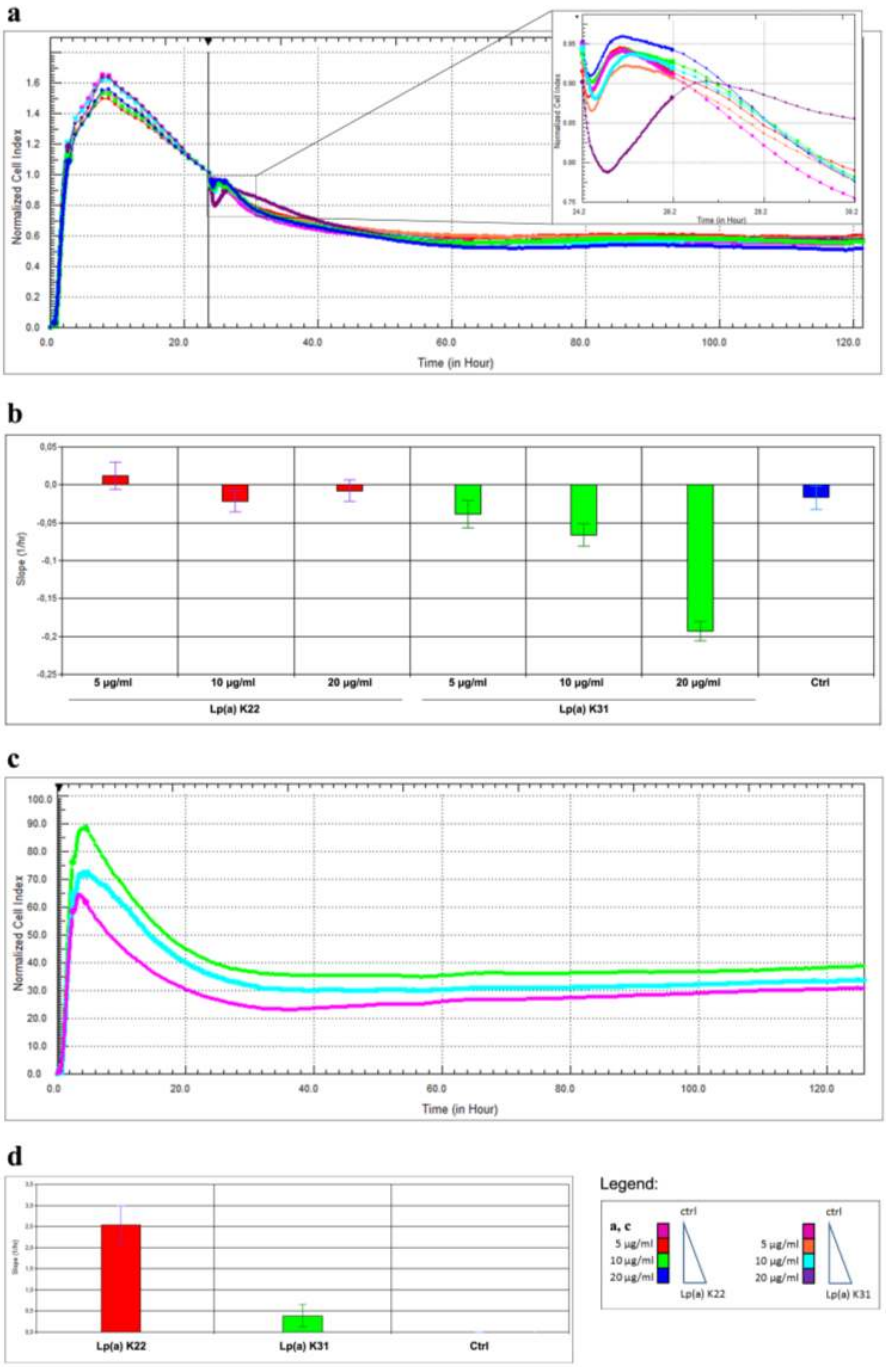

Fig. 5. Real Time Cell Analysis (RTCA) of THP-1 cells exposed to Lp(a) (a) Full Normalized Cell Index (NCI) profile, with normalization performed by dividing the Cell Index by its value at the last time point before treatment. Cells were treated at 24 h (a) or 2 h (c). A magnification box of the 24.2-30.2 h interval is shown; (b) Histogram representation of slope values for the two isoforms (K22 and K31) at different concentrations in the case of a treatment performed at 24 h; (d) Histogram representation of slope values for the two isoforms (K22 and K31) at 10 µg/ml in the case of a treatment performed at 2 h

Colour legend: See figure

4. DISCUSSION

Despite the demonstrated role of Lp(a) as a cardiovascular risk factor, the biological significance of its different isoforms in the pathogenesis of atherosclerosis has not been clarified yet. A direct consequence is the impossibility to formulate appropriate clinical hypotheses and studies. Cellular studies are complicated by the fact that demonstrated and putative Lp(a) receptors include several different molecules, among which the low-density lipoprotein receptor (LDLR) [14], the low-density lipoprotein receptor-related protein (LRP) [15], the very low density lipoprotein receptor (VLDLR) [16], megalin [17], ASGPR [18] and SR-BI [19]. All of them are differentially represented in different cells types, making the measurement of cell impedance kinetics ideal to have information about the global combined effect of Lp(a) on basic cell features, like morphology, adhesion and proliferation. In this work, we have analysed for the first time the change in impedance kinetics occurring upon treatment with Lp(a) in cell lines which represent key models of Lp(a) metabolism and atherogenesis (Table 2). Cell impedance is a relatively new parameter that can be useful to characterize the behavior of adherent cells and, although the resulting data require accurate interpretation, it has valuable advantages compared to other analyses, such as the possibility to have a label-free, real-time and continuous control of the cell state. Moreover, the high sensitivity of cell impedance measurements allows the detection of small differences in cell response upon treatment with different isoforms of Lp(a). These differences, that could remain elusive using classical methods for cell analysis or that would require receptors overexpression, may help in the definition of a threshold for Lp(a) pathogenicity in terms of apo(a) size, which could greatly improve the clinical management of Lp(a) monitoring.

Lp(a) catabolism is operated mainly by the liver, possibly through involvement of the apo(a) moiety [20]. Internalization of Lp(a) in HepG2 cells was documented in different studies [21,22], but without distinction in apo(a) size. In fact, the early NCI increase we observed can be interpreted as a change in morphology, possibly due to internalization of Lp(a) particles and, significantly, it depends both on Lp(a) concentration and MW, with the smaller Lp(a) isoform exerting a more pronounced effect. This observation could be relevant if confirmed *in vivo*, as it might indicate a preferential cell processing for small isoforms by hepatic cells.

Deposition and accumulation of Lp(a) particles in the atherosclerotic blood vessel wall exacerbates atherosclerotic plaques, by triggering different responses in the “resident” cells, such as macrophages, endothelial and smooth muscle cells. For example, Lp(a) promotes adhesion and transendothelial migration of inflammatory cells inducing expression of adhesion molecules in both endothelial cells and macrophages [23]. Direct binding of apo(a) to β2-integrin Mac-1 [24] was stronger when recombinant forms of small apo(a) isoforms were used, confirming a previous study in which small Lp(a) isoforms showed a higher affinity for the cell surface of THP-1 monocytes [25]. Our RTCA on THP-1 macrophages, in fact, shows an opposite effect exerted by the low and the high MW Lp(a) isoforms: particularly, in the medium- and long-term, K22 diverges from K31 and the control, showing a consistent decrease of NCI. One possible reason for the decrease in NCI is the reduction of cell number due to apoptosis. Indeed, it was recently found that Lp(a) induced apoptosis in endoplasmic reticulum stressed murine macrophages through the CD36-TLR2 pathway [26]. According to our data, we can hypothesize that the K22 isoform could be more powerful than the K31 one in triggering the apoptosis pathway of lesional macrophages, a mechanism which has been described as relevant in promoting the development of the necrotic core in advanced atherosclerotic lesions [27,28].

Besides inducing the increase in the expression of adhesion molecules [23], Lp(a) was found to exert a stimulatory effect on growth and migration of human vascular endothelial cells [29,30], even using different Lp(a) isoforms [31], but also to induce ROS production and apoptosis in human endothelial cells [32]. Our findings in RTCA support an earlier stimulation of proliferation by K22 compared to K31. Time could therefore be a critical factor to consider in analysing the effect of Lp(a) on this cell type and could have an *in vivo* correlate in the observed higher impact of small isoforms in promoting cardiovascular risk.

Smooth Muscle Cells (SMCs) were found to comprise at least 50% of total foam cells in human coronary artery atherosclerosis [33]. A number of studies reports an increased proliferation of SMCs caused by Lp(a) [34-37] in

Table 2. Summary of Lp(a) effects on normalized Cell Index profiles of cell impedance

	K22 Lp(a)	K31 Lp(a)
HepG2	Higher increase	Lower at the end of treatment
THP-1	Lower increase in NCI Divergence towards lower NCI values Higher reduction of NCI at higher concentrations Sudden increase in NCI at 20 µg/ml, 84 h	Higher increase in NCI Divergence towards higher NCI values
HUVEC	Higher than control, starting at 58 h	Higher than control only at > 96 h
HASMC	Higher than control at all concentrations Reduction starting at 68 h	Reduction at 20 µg/ml (2 h), then realignment to control

a dose- and time-dependent manner. Specifically, SMC proliferation was attributed to the KIV-9 domain of apo(a) [35,36], but also to the lipidic components of Lp(a) [34,37]. However, none of these studies considered a potential role of apo(a) size. Our data suggest that the K22 isoform can induce a more pronounced stimulation of proliferation than K31, when treatment is performed in the initial 2 h after seeding. Overall, the effect of K31 is also more inhibitory than K22 when treatment is performed at 24 h after seeding. As SMCs appear to be the origin of the majority of foam cells in the human atherosclerotic plaque [33], our data support the hypothesis that small Lp(a) isoforms could be more active in promoting SMCs proliferation.

5. CONCLUSION

In summary, time-wise, Lp(a) exerts an earlier effect on HepG2, THP-1 and HASMC cells than on HUVEC cells. Regarding differences in isoforms, K22 Lp(a) induces a consistent early and high increase in NCI compared to K31 Lp(a) in HepG2, HUVECs and HASMCs. An opposite behaviour is instead measured in THP-1 cells, where K31 is the most effective isoform in increasing NCI. These data suggest that Lp(a) can alter both morphology and proliferation of exposed cells, which are key parameters of the atherosclerosis process, in a complex way, very much dependent on the cell type and time analyzed, and on the size of the isoform considered. Interestingly, the effects observed are all consistent with the hypothesis that small Lp(a) isoforms could be more active than large ones in stimulating endothelial and SMCs proliferation and in inducing apoptosis of macrophages, typical features of atherogenesis.

For the first time, we showed that also long-term intervals need to be considered when analysing the effect of Lp(a) on cells and that cell impedance kinetic profiles can be a useful tool to identify the global effects of Lp(a) isoforms on critical cell types. These data deserve further studies by molecular and morphological approaches and they could be relevant in order to design novel treatment strategies.

ETHICAL APPROVAL

All the plasma samples were obtained from donors affiliated to the Immunoheamatology 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 thank Dr. Paola Isernia and Prof. ssa Laura Salvaneschi from the Servizio Immunoematologia e Medicina Trasfusionale (S.I.M.T.) of IRCCS San Matteo Foundation, Pavia, for providing plasma bags. Funding for this work have been provided by the grant: POR Competitività 2007-2013 – Asse 1, Linea di intervento 1.1.1.2 - Azione A, project number 42657763. We acknowledge Dr. Angela D'Angelo for her help in setting up the western blotting technique for Lp(a) and Dr. Giuseppe De Rosa and Dr. Paolo Giordano (ABS-Advanced Biomedical Systems) for useful discussion.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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