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Lipoprotein (a) stimulates mitogen activated protein kinase in human mesangial cells

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Abstract Evidence suggests an important role of elevated serum lipoproteins in the progression of renal glomerulosclerosis. We report here that lipoprotein (a) (Lp(a)) increased phosphorylation and activity of mitogen activated protein kinase (MAPK) in human mesangial cells. When protein kinase C (PKC) was depleted by long-term incubation with the phorbol 12-*O*-myristate 13-acetate the effect of Lp(a) on MAPK activation was completely inhibited. Forskolin, a stimulator of the adenylyl cyclase, and dibutyryl-cAMP reduced the effect of Lp(a) on MAPK phosphorylation and activation. We conclude that Lp(a) stimulates the MAPK cascade via activation of PKC and that activation of protein kinase A counteracts Lp(a) induced MAPK activation in human mesangial cells.

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Key words: Lipoprotein (a); Mesangial cell; Mitogen activated protein kinase; Signal transduction; Protein kinase C; Protein kinase A

1. Introduction

Lipoprotein abnormalities are a characteristic feature of chronic renal insufficiency [1]. It has been suggested that alterations of the lipoprotein metabolism contribute to the progression of glomerulosclerosis [2,3]. Biochemical and histological similarities between atherosclerosis and glomerulosclerosis have been proposed [4], and in animal studies lipid lowering treatment was shown to be beneficial in slowing the progression of renal disease [5,6]. Lipoprotein (a) (Lp(a)) is recognized as an independent risk factor of atherosclerosis and Lp(a) serum concentrations were found to be elevated in patients with nephrotic syndrome. Inflammation or damage of the glomerular barrier may facilitate access of macromolecules to the mesangium. This influx may result in cytokine activation and secondary proliferative responses. In vitro experiments showed that low density lipoprotein (LDL) and Lp(a) bind to mesangial and epithelial cells [7,8]. LDL stimulates proliferation of these cells [9], whereas Lp(a) stimulates proliferation and increases mRNA levels for c-myc and c-fos in rat mesangial cells [10]. LDL increases cytosolic calcium ($[Ca^{2+}]_i$) in vascular smooth muscle cells and rat mesangial cells [11–13]. Furthermore, LDL is growth promoting in human skin fibroblasts of patients with familial hypercholesterolemia lacking the LDL receptor, suggesting an independent effect of the classic LDL receptor [14]. LDL activates the $p42^{ERK2}/p44^{ERK1}$ kDa mitogen activated protein kinase (MAPK) in vascular smooth muscle cells, fibroblasts, and rat mesangial cells [15,16]. We have recently shown that Lp(a) increases $[Ca^{2+}]_i$ and inositol 1,4,5-trisphosphate (IP_3) levels by a pertussis toxin (PTX) sensitive G_i coupled receptor and stimulates proliferation of human mesangial cells (in press). The growth stimulatory effect of Lp(a) suggests that signal transduction through the MAPK is one of the downstream events of Lp(a). In the present study we investigated the effect of Lp(a) on MAPK activity. In addition, we examined the effect of adenylyl cyclase activation on Lp(a) induced MAPK activation, since substances which increase cAMP are known to counteract proliferative and vasoconstrictive effects in mesangial cells [17].

2. Materials and methods

2.1. Materials

Enhanced chemiluminescence (ECL) reagents, cAMP assay, [γ-^{32}P]ATP, secondary horseradish peroxidase (HRP) labeled antibodies and X-ray films were obtained from Amersham Inc. (Little Chalfont, UK). MAPK assay was from Stratagene (La Jolla, CA, USA), RPMI 1640 medium and fetal calf serum (FCS) were from Gibco (Gaithersburg, MD, USA). Nitrocellulose membrane (pore size 0.2 μm) was from Schleicher and Schuell (Dassel, Germany), anti-phosphotyrosine antibody was purchased from ICN (Costa Mesa, CA, USA). Anti-$p42^{ERK}$ antibody and forskolin were from Calbiochem (La Jolla, CA, USA), HRP labeled protein markers and the antibody raised against dually phosphorylated MAPK ($p42^{ERK2}/p44^{ERK1}$) were from New England Biolabs (Beverly, MA, USA). Phorbol 12-*O*-myristate 13-acetate (PMA) and dibutyryl cAMP (db-cAMP) were from Sigma (St. Louis, MO, USA).

2.2. Isolation of Lp(a)

Lp(a) was purified from the regenerate fluid of a dextran sulfate column based LDL-apheresis system (Kanekafuchi MA 01 – Liposorber LA 15) as described [18]. Apo(a) phenotypes were determined as an S2/S4 phenotype with the nomenclature of Utermann [19] with a number of 22/31 kringle 4 repeats. For all experiments Lp(a) was isolated from a single donor. Prior to use Lp(a) was extensively dialyzed against phosphate buffered saline (PBS).

2.3. Cell culture

Human mesangial cells were isolated by the sequential sieving technique from patients undergoing tumor nephrectomy. All experiments were performed at passages 3–6. Mesangial cell cultures were identified by morphological and immunological characteristics such as positive staining for actin and negative staining for factor VIII and cytokeratin. To exclude fibroblast contamination cells were grown for 2 weeks in L-valine deficient RPMI 1640 supplemented with 10% FCS, 10 mM HEPES, pH 7.4, 1 mM sodium pyruvate, 100 U/ml penicillin/streptomycin. Culture media were replaced three times per week.

2.4. Cell stimulation and preparation of cell extract

Cells were grown until subconfluence in media supplemented with 10% FCS. Cells were washed once and made quiescent by a 48 h incubation in serum-free medium. Lp(a) was added at different time

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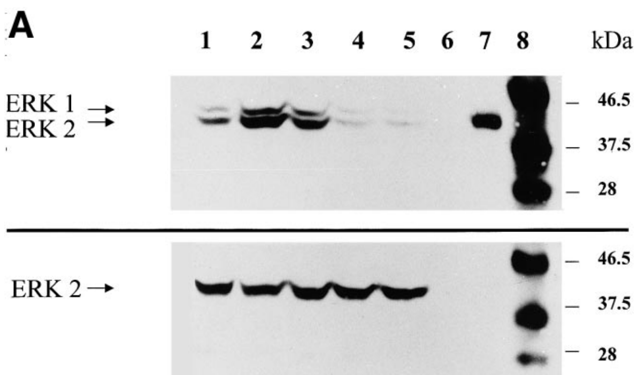
points. The incubations was terminated by washing cells twice with ice-cold PBS. After scraping the cells were pelleted by centrifugation and lysed in a buffer containing 1% Triton X-100, 0.32 M sucrose, 5 mM EDTA, 10 mM Tris-HCl (pH 8.0), 1 mM PMSF, 2 mM DTT, 10 mM sodium pyrophosphate, 200 mM sodium orthovanadate.

2.5. Measurement of MAPK activity

MAPK activity was measured by a commercially available in vitro kinase assay using Phas I protein as a substrate. Cell lysates containing 20 μg protein were incubated at 30°C for 10 min in 4 μl of 10× kinase buffer (250 mM HEPES, 100 mM magnesium acetate, 500 μM ATP), 2 μCi [γ-^{32}P]ATP and 10 μg Phas I protein and H_2O to a final volume of 40 μl. The reaction was stopped by adding 5 μl of 0.25 M H_3PO_4. 20 μl samples were spotted onto phosphocellulose sheets and washed four times with 75 mM H_3PO_4. Thereafter, the sheets were rinsed briefly in 95% (v/v) ethanol and dried on a paper towel. Radioactivity of the sheets was measured by determination of Cerenkov radiation. Controls lacking the cell lysates, Phas I or both were run in parallel. Activities are expressed as phosphotransfer per minute per mg protein.

2.6. Immunoblotting of MAPK

Cell lysates were protein matched (150 μg/lane) and separated by SDS-PAGE and electrotransferred to nitrocellulose sheets. Western blotting was carried out as described recently [20]. Antigen-antibody complexes were visualized using secondary HRP conjugated antibodies and the ECL system.



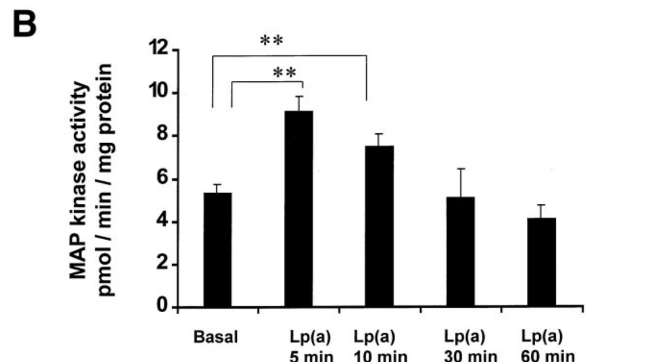


Fig. 1. A: Effect of Lp(a) on phosphorylation of MAPK in human mesangial cells. Cells were incubated with Lp(a) (10 μg/ml) for the indicated time periods. After lysis of the cells equal amounts of protein (150 μg) were immunoblotted with an anti-phospho-MAPK antibody. After stripping the same blot was reprobed with an anti-p42^{ERK2} antibody. Lane 1: basal; lane 2: Lp(a) 5 min; lane 3: Lp(a) 10 min; lane 4: Lp(a) 30 min; lane 5: Lp(a) 60 min; lane 6: blank; lane 7: positive control consisting of phosphorylated p44^{ERK1}; lane 8: molecular weight marker. B: Effect of Lp(a) on activation of MAPK in cultured human mesangial cells. The activity of MAPK was measured by in vitro kinase assay. Cells were incubated with Lp(a) (10 μg/ml) for 5, 10, 30 and 60 min. Values are mean ± S.D. ($n = 4$). Asterisks indicate significant differences (**$P < 0.001$).

2.7. Measurement of cAMP

30 000 cells were plated on 36 mm culture dishes and allowed to reach subconfluence over 7 days. For measurement of intracellular cAMP, the medium was replaced by serum-free medium containing the indicated concentration of forskolin or Lp(a) for 1 h. The stimulation was terminated by aspiration of the medium and the addition of ice-cold 65% ethanol. The cAMP content of the samples was determined using a radioimmunoassay and was performed according to the protocol provided by the manufacturer.

2.8. Miscellaneous

Protein was measured according to the method of Bradford [21] using bovine serum albumin as a standard. The data presented are means ± S.D. Statistical significance was calculated using Student's *t*-test for paired values.

3. Results

3.1. Effects of Lp(a) on MAPK phosphorylation and activation

To demonstrate activation of MAPK we performed immunoblots with a specific anti-phospho-MAPK antibody, which detects the dually phosphorylated form of p44^{ERK1} and p42^{ERK2}. Lp(a) (10 μg/ml) caused an increase in p42^{ERK2}/p44^{ERK1} phosphorylation between 5 and 10 min incubation. After 30 min and 60 min incubation phosphorylation of p42^{ERK2}/p44^{ERK1} declined to basal levels (Fig. 1A). Reprobing of the immunoblots with the p42^{ERK2} specific antibody revealed equal loading of the lanes.

To measure MAPK activity cells were incubated with Lp(a) (10 μg/ml) for 5, 10, 30 and 60 min. MAPK was measured in homogenates using Phas I protein as substrate. Lp(a) induced MAPK activity increased significantly ($P < 0.001$) from a basal level of 5.35 ± 0.36 pmol/min/mg protein to 9.13 ± 0.67 (5 min) and 7.51 ± 0.53 (10 min). MAPK activities were not different from the basal level at 30 min (5.08 ± 1.34 pmol/min/mg protein) and 60 min (4.10 ± 0.61 pmol/min/mg protein). The effect of epidermal growth factor (EGF) (Fig. 2) was considerably higher compared to Lp(a) (EGF 22.72 ± 1.81 pmol/min/mg protein, Lp(a) 18.84 ± 0.8 pmol/min/mg protein; $P < 0.001$ vs. control).

3.2. Role of protein kinase C (PKC) in Lp(a) induced MAPK phosphorylation

When PKC was depleted by overnight treatment of the cells

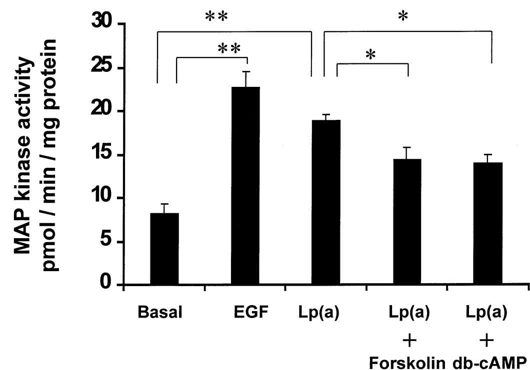


Fig. 2. Effect of forskolin and db-cAMP on Lp(a) induced activation of MAPK in human cultured mesangial cells. The activity of MAPK was measured by in vitro kinase assay. Cells were incubated with Lp(a) (10 μg/ml) or EGF (10 ng/ml) for 10 min or were preincubated with forskolin (100 μM) or db-cAMP (1 mM) for 30 min prior to the addition of Lp(a). Values are mean ± S.D. ($n = 4$). Asterisks indicate significant differences (*$P < 0.05$; **$P < 0.01$).

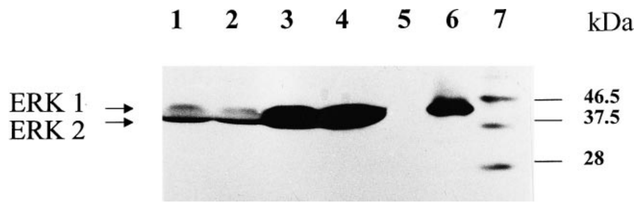


Fig. 3. Influence of PKC on Lp(a) induced MAPK phosphorylation. After lysis of the cells equal amounts of protein (150 μg) were immunoblotted with an anti-phospho-MAPK antibody. Cells were stimulated with Lp(a) (10 μg/ml) (lane 3) or PMA (0.5 μM) (lane 4) for 30 min. To deplete cells from PKC, cells were preincubated for 12 h with PMA (0.5 μM). Then, the cells were incubated with Lp(a) (lane 1) or PMA (lane 2). Lane 5: blank; lane 6: positive control consisting of phosphorylated $p44^{ERK1}$; lane 7: molecular weight marker.

with PMA (100 nM), neither Lp(a) (10 μg/ml) nor PMA (500 nM) caused a significant increase in phosphorylation of MAPK (Fig. 3), whereas the effect of EGF remained unchanged (data not shown). Thus, activation of MAPK by Lp(a) appears to depend on activation of PKC.

3.3. *Effects of cAMP on Lp(a) induced phosphorylation of MAPK*

Substances known to increase the cAMP level are known to inhibit proliferation of mesangial cells and phosphorylation of mesangial MAPK [22]. As illustrated in Fig. 4, Lp(a) (10 μg/ml) had no significant effect on cAMP concentration, whereas forskolin (50 μM), a direct stimulator of adenylyl cyclase, increased the cellular cAMP level.

Preincubation of cells with forskolin (100 μM) or db-cAMP (1 mM) inhibited Lp(a) (10 μg/ml) induced MAPK phosphorylation (Fig. 5). As shown in Fig. 2 forskolin (100 μM) and db-cAMP (1 mM) reduced MAPK activity only partially from 18.84 ± 0.79 pmol/min/mg protein to 14.39 ± 1.42 pmol/min/mg protein and 13.93 ± 1.04 pmol/min/mg protein respectively ($P < 0.01$ vs. Lp(a)).

4. Discussion

The interaction of Lp(a) with glomerular mesangial cells has been investigated. It was shown that Lp(a) is taken up by rat and human mesangial cells through a typical feature of a receptor mediated process, i.e. Michaelis-Menten kinetics and saturability, suggesting distinct receptor sites for Lp(a). Furthermore, glomerular deposition of apolipoprotein (a) and functional properties of Lp(a) have been shown such as stimulation of cell proliferation and expression of c-myc and c-fos in rat mesangial cells [10,23,24]. The growth stimulatory effect of Lp(a) in human mesangial cells suggests that the activation of MAPK by Lp(a) might be an important mechanism in Lp(a) signaling. Therefore, in the present study, we assessed the effect of Lp(a) on MAPK activity in human mesangial cells and the influence of PKC and protein kinase A (PKA) activation in this process.

MAPKs play a key role in cellular proliferation and differentiation [25]. Incubation of mesangial cells with Lp(a) phosphorylated mainly $p42^{ERK2}$ and $p44^{ERK1}$ and rapidly stimulated phosphorylation of exogenous Phas I protein. The time course of MAPK phosphorylation was similar to those reported for endothelin I and angiotensin II in mesangial cells [26,27]. We have thus concluded that Lp(a) stimulation of human mesangial cells can increase $p42^{ERK2}/p44^{ERK1}$ phosphorylation. Other lipoproteins such as LDL, mildly oxidatively modified and oxidatively modified LDL and high density lipoprotein (HDL) also activated $p42^{ERK2}$ [15]. These effects were mediated by elevation of $[Ca^{2+}]_i$ and activation of PKC.

Our finding that Lp(a) did not activate MAPK in PKC depleted cells suggests that PKC is involved in Lp(a) induced MAPK activation. Other experiments with human skin fibroblasts have shown that lipoproteins such as LDL and HDL_3 stimulated PKC by a phosphoinositide specific pathway [28].

It is known that agents which increase the cAMP concentration counteract the effects of vasoconstrictive and proliferative agents in mesangial cells [29], whereas in PC12 and COS-7 cells cAMP stimulated MAPK [30,31]. We hypothesized that the effects of Lp(a) might also be inhibited by an increase in cAMP. Preincubation with forskolin or db-cAMP inhibited the Lp(a) induced stimulation of MAPK. However, the inhibitory effect was incomplete. MAPK activity measured by in vitro kinase assay did not return to control level. Thus, forskolin and db-cAMP only partially inhibited Lp(a) signaling. Other investigators have shown that cyclic nucleotides such as cAMP and cGMP inhibited endothelin I induced activation of MAPK at different steps [17], the former downstream of PKC and the latter prior to PKC activation. Growth factor induced stimulation MAPK is also inhibited by cAMP by interfering with Raf-1 activation [22]. Since PKC can directly phospho-

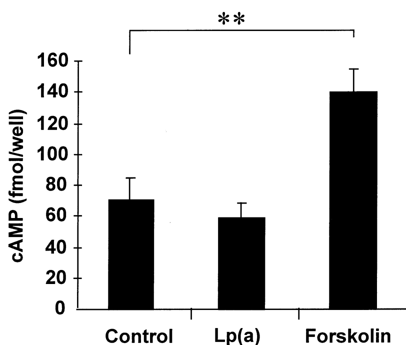


Fig. 4. Effect of Lp(a) and forskolin on the cellular cAMP concentration. Cells were treated with 10 μg/ml Lp(a) and 50 μM forskolin for 1 h and cAMP accumulation was measured as described in Section 2. Values are mean ± S.D. ($n = 4$). Asterisks indicate a significant difference ($^{**}P < 0.01$).

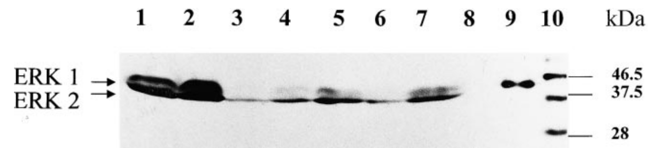


Fig. 5. Effect of forskolin and db-cAMP on Lp(a) induced MAPK phosphorylation. Cell lysates were matched for protein and immunoblotted with an anti-phospho-MAPK antibody. Lanes 1 and 5: control; lane 2: Lp(a) (10 μg/ml) for 30 min; lane 3: preincubation of the cells for 30 min with forskolin (100 μM) followed by incubation with Lp(a) (10 μg/ml) for 30 min; lane 4: preincubation for 30 min with db-cAMP (1 mM) followed by incubation with Lp(a) (10 μg/ml) for 30 min; lane 6: incubation for 30 min with forskolin; lane 7: incubation for 30 min with db-cAMP; lane 8: blank; lane 9: positive control of phosphorylated $p44^{ERK1}$; lane 10: molecular weight markers.

rylate Raf-1 [32], we speculate that cAMP also inhibits activation of Raf-1 by Ras in our experiments.

Taken together, we have shown for the first time that Lp(a) stimulates MAPK in a human primary cell. Whether these effects contribute to the pathophysiology of glomerulosclerosis requires further investigation.

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