Lipoprotein(a) induces glomerular superoxide anion production

S. Greiber¹, M. Kreusel¹, H. Pavenstädt¹, P. Schollmeyer¹ and C. Wanner²

Department of Medicine, Division of Nephrology, University Hospital of ¹Freiburg and ²Würzburg, Germany

Abstract

Background. Lipoprotein(a) (Lp(a)) is considered to accelerate glomerular injury in various forms of renal disease. Several tissue culture studies suggested that biological effects of Lp(a) are inhibitable by oxygen radical scavengers. Since reactive oxygen metabolites (ROM) are important mediators of renal disease, we studied the effects of native and oxidized Lp(a) on generation of the ROM superoxide anion in isolated glomeruli and compared them with the effects of native (nLDL) and oxidized LDL cholesterol (oxLDL).

Methods. The effect of native and oxidized Lp(a) and LDL on ROM production in isolated rat glomeruli was investigated with a lucigenin chemiluminescence assay.

Results. Native Lp(a) caused a moderate, dose dependent stimulation of glomerular ROM production: Maximum ROM production to 159±9% of control glomeruli was induced by nLp(a) 20 μg/ml. Lp(a)-induced chemiluminescence was completely inhibited by the cell permeable oxygen radical scavenger Tiron (10 mM). Oxidized Lp(a) (20 μg/ml) caused a more pronounced stimulation of ROM production to 204±12% of control values. Interestingly, only oxLDL, but not nLDL had a significant effect on glomerular ROM production (oxLDL 50 μg/ml: 192±19% of control). Lp(a) stimulated ROM production was completely inhibited by the protein kinase C inhibitor bis-indolyl maleide (BIM): BIM 10⁻⁸ M inhibited 52±3%, BIM 10⁻⁹ M inhibited 94±5% of Lp(a)-induced ROM production. ROM production was also inhibited, when intracellular CAMP levels were elevated by forskolin.

Conclusion. Lp(a) and oxLp(a) induce the activation of ROM in glomeruli by a pathway that is sensitive to inhibition of protein kinase C and elevation of intracellular CAMP levels.

Key words: superoxide anion; glomerulus; protein kinase C; cyclic AMP; LDL cholesterol; forskolin; bis indolyl maleide
that increased levels of intracellular cyclic adenosine monophosphate (cAMP) can suppress the burst of ROM generation in inflammatory cells [13,14] and that this pathway is also present in glomeruli [15,16]. In view of these data, we also tried to determine whether Lp(a) stimulated ROM production was inhibited by elevating intracellular cAMP levels with forskolin.

Subjects and methods

Preparation of glomeruli

Glomeruli were isolated from about 50 rats following established protocols [17]. Briefly, following anaesthesia, the aorta of male Sprague–Dawley rats (200–250 g) was cannulated and the kidneys were perfused with cold phosphate-buffered saline (PBS) with 5 mM glucose until paled. Kidneys were removed and placed into 4 °C PBS. All subsequent steps were carried out at 4 °C. Kidneys were decapsulated and the medulla was removed. The cortex was squeezed through sieves with 150-, 105-, and 50-μm openings. Tissues retained on the last sieve were collected with PBS and centrifugated at 1000 r.p.m. for 5 min. The supernatant was removed to eliminate small fragments, the remaining glomeruli (Glm) weighed and resuspended to a concentration of 25 mg/ml.

As published previously, this protocol generates glomeruli with intact cells [17]. The purity of each preparation was assessed by counting Glm under light-microscopy. There only was a minor contamination with tubular cells.

Preparation of lipoproteins

Preparation and Cu-oxidation of lipoprotein(a) and LDL-cholesterol was carried out as previously described [6,18]. Homogeneity and stability of lipoproteins was tested frequently by agarose gel electrophoresis (REP-HDL-plus cholesterol electrophoresis, Helena Diagnostics, Hartheim, Germany). Native Lp(a) was found to migrate in the pre-beta region and was free of LDL, VLDL and HDL protein contamination. To exclude aggregation and to confirm purity of the Lp(a) preparations, fast protein lipoprotein chromatography was performed with a chromatography system from Pharmacia (Uppsala, Sweden) according to the method of März et al. [19]. Lp(a) was from two donors and characterized as S1/S3 and S1/S2 subtype by immunoblotting [20]. Both isoforms belong to the low-molecular-weight isoforms which have been attributed with an enhanced atherosclerotic risk profile. As in our previous cell culture studies [6,18], we did not detect a difference between the biological effects of the two isoforms. Lp(a) from six different preparations was used in the present study. Lp(a) preparations were free of relevant endotoxin contaminations [18].

Measurement of ROM generation

The content of each reaction tube was a mixture of 0.25 mM lucigenin, 7 mM diethyl thiocarbamic acid (DTC), approximately 7000 Glm in 200 μl, and PBS to make the final volume 1 ml. DTC, an inhibitor of endogenous CuZn-superoxide dismutase was added to inhibit the reaction of the ROM O₂⁻ to OH⁻ and H₂O₂, the latter being ROM which are not detected by the lucigenin assay [21]. ROM were quantitated by chemiluminescence in a LB 9500 Luminometer at 37 °C with intermittent mixing. With a photomultiplier, the luminometer converts photons into an electric current. Following the preincubation of a tube for 5 min (with or without lipoproteins), baseline chemiluminescence was recorded. In some experiments, Glm were preincubated with the PKC inhibitor bisindolyl maleimide or forskolin for 15 min before lipoproteins were added. Chemiluminescence was recorded thereafter every 10 min with an integration for 30 s until a peak plateau was reached.

Results

A typical chemiluminescence response of control and Lp(a)-treated Glm is shown in Figure 1. After a lag period, chemiluminescence increased in control and Lp(a)-treated Glm. Peak plateau levels were reached after 80–90 min, chemiluminescence declined thereafter. There was no statistical difference for the amount of time to reach peak plateau levels between control
Table 1. Effects of native or oxidized Lp(a) and LDL on superoxide generation in Glm

<table>
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<tr>
<th></th>
<th>Control</th>
<th>nLP(a) [20 mg/ml]</th>
<th>oxLP(a) [20 mg/ml]</th>
<th>nLDL [50 mg/ml]</th>
<th>oxLDL [50 mg/ml]</th>
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<tr>
<td>O$_2^-$ generation</td>
<td></td>
<td></td>
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<td>(nmol/10$^5$ Glm/min)</td>
<td>2.59 ± 0.17</td>
<td>4.10 ± 0.51*</td>
<td>4.88 ± 0.56*</td>
<td>3.58 ± 1.50</td>
<td>5.30 ± 1.09</td>
</tr>
<tr>
<td>Time to reach peak</td>
<td>80.2 ± 3.14</td>
<td>88.2 ± 6.92*</td>
<td>87.8 ± 3.11</td>
<td>84.3 ± 4.8</td>
<td>85.6 ± 4.8</td>
</tr>
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To calculate net ROM generation, peak luminiscence data from control and lipoprotein stimulated glomeruli were transformed using a standard curve generated by the xanthin/xanthinoxidase reaction. Data from 6–15 experiments with control and lipoprotein stimulated glomeruli were evaluated. A significant stimulation of ROM generation vs control is indicated (*P < 0.001; #P < 0.025 by unpaired $t$ test). The time course of ROM production was not different between control and lipoprotein-stimulated glomeruli.

and lipoprotein treated Glm (Table 1). Chemiluminescence was rapidly inhibited by addition of Tiron 10 mM, a cell permeable scavenger of ROM (Figure 1).

Initial experiments revealed that no significant stimulation of ROM production occurred when Glm were exposed to native or oxidized Lp(a) concentrations below 20 μg/ml (figure 2). Treatment of Glm with native Lp(a) (20 μg/ml) caused a moderate, but significant increase in peak plateau luminescence (Figure 2), which was not further stimulated by higher concentrations of Lp(a). The effects of oxidized Lp(a) (oxLp(a)) on ROM production were more pronounced than the effects of nLp(a) (Figure 2). Glm were also exposed to native (nLDL) and oxidized (oxLDL): Interestingly, nLDL had no significant effect on ROM production whereas the effects of oxLDL (50 μg/ml) were comparable to the effects of oxLp(a) (Figure 3). Net ROM production of the respective lipoprotein concentrations causing a maximal stimulation was calculated using a standard curve generated by the xanthin/xanthin oxidase reaction (Table 1). To test whether oxLp(a) and oxLDL might have additive effects on ROM production, we compared the effects of each of the two lipoproteins to the effects of combined stimulation. In these experiments (n = 3), oxLp(a)-induced chemiluminescence was not increased by concomitant incubation with oxLDL, suggesting a common pathway of stimulation.

Inhibition of PKC by bisindolyl maleimide (BIM) 10$^{-6}$M caused a significant reduction of Lp(a)-stimulated ROM production, this effect was further enhanced by BIM 10$^{-5}$M (Figure 4). ROM production was also suppressed by the diterpene forskolin, an effective activator of adenylate cyclase, but not by the inactive forskolin derivate 1,9 dideoxyforskolin (Figure 4). DMSO, which was used as a solvent for forskolin and BIM, had no effect on chemiluminescence (data not shown).

**Discussion**

In this study we demonstrate for the first time that Lp(a), and especially in its oxidized form, induces ROM production in intact glomeruli. ROM production was also induced by oxidized, but not by native LDL.
We also found, that Lp(a) induced ROM production was sensitive to inhibition of PKC with BIM and to elevation of intracellular cAMP levels with forskolin. In the following section, we will discuss the relevance of these findings for the pathophysiology of glomerular disease.

Major manifestations of glomerular disease are proteinuria, altered GFR and, depending on the type of disease, morphological changes such as increased matrix production and, ultimately, glomerular sclerosis. Sato et al. [5] demonstrated that glomerular Lp(a)-deposits correlated with a more severe cause of renal disease, i.e. more severe proteinuria and a more rapid decline of renal function. Clinical observations, consistent with experimental studies, have come to the conclusion that lipid abnormalities are modulators, rather than primary initiators of renal disease [1]. In experimental studies, LDL-cholesterol was shown to modify several pathophysiological mechanisms that could ultimately lead to the morphological changes described above: LDL-cholesterol in concentrations below 50 µg/ml stimulated mesangial cell proliferation and expression of early genes c-fos and c-jun [23,24], whereas higher concentrations had antiproliferative effects. LDL enhances the expression of the leukocyte-chemoattractant MCP-1 and induces the production of the extracellular matrix protein fibronectin [25]. It is attractive to speculate that lipoprotein-induced production of ROM by resident glomerular cells might be the unifying mechanism for mesangial proliferation and enhanced expression of leukocyte-chemoattractants: ROM stimulate growth and expression of immediate early genes c-fos and c-jun [26] which serve as nuclear transcription factors [26]. Binding of the fos/jun transcription factor to DNA is also under the control of the cells redox status [27]. Satirano et al. [28] have shown that in mesangial cells, enhanced protein-expression and mRNA of MCP-1 and monocyte colony-stimulating factor-1 (CSF-1) in response to TNF-α and aggregated IgG was attenuated by oxygen radical scavengers. They concluded that generation of ROM, possibly by a NADPH dependent oxidase, is involved in the induction of MCP-1 and CSF-1 genes.

Although studies in vascular smooth muscle cells initially advanced the idea that the prominent effect of Lp(a) was induction of cellular proliferation [29], mesangial cells were shown to respond differently to Lp(a). We demonstrated a biphasic proliferative response in rat mesangial cells that were stimulated with Lp(a): Low levels of Lp(a) induced DNA synthesis and elevated mRNA levels of immediate early response genes c-fos and c-myc [6]. In higher concentrations, Lp(a) suppressed DNA-synthesis, inhibited cell growth, and finally displayed overt cytotoxic effects. In studies with human mesangial cells, exclusively inhibitory effects of native Lp(a) and oxLp(a) on DNA-synthesis were observed [18]. These findings are reminiscent to the inhibitory effects of oxLDL on DNA synthesis in cultured mesangial cells [30]. We believe, that the effects of oxidized lipoproteins on the glomerulus, i.e. antiproliferative and toxic effects, most likely due to increased ROM-production, are more relevant than the effects of unmodified LDL: nLDL, but not nLp(a), oxLp(a) and oxLDL is taken up and degraded by mesangial cells, hence, an effective clearing mechanism exists [18]. Lp(a), oxLp(a) and oxLDL are avidly bound by the mesangial matrix [18] and could start a vicious cycle by further enhancing autooxidative processes. In regard to the effects of Lp(a) on extracellular matrix production, we could show that Lp(a) actually suppresses production and mRNA of the extracellular matrix protein fibronectin in cultured mesangial cells (unpublished observation). This finding could also be a consequence of increased ROM production by glomerular cells in response to Lp(a): In perfused kidneys, Kashihara et al. [31] could demonstrate a strong decrease in the de novo synthesis of proteoglycans and a slight decrease of type IV collagen and laminin synthesis in response to the xanthin/xanthinoxidase system.

Besides direct effects on mesangial cell pathology, Lp(a)-induced ROM production might have other consequences: (1) ROM might participate in glomerular basement degradation, ultimately leading to proteinuria; (2) ROM might have profound effects on podocyte morphology, leading to foot process effacement with loss of the slit membrane barrier and subsequent development of proliferative glomerular lesions (for review see [10]).

Previous studies in Glm and mesangial cells employing PMA, a strong activator of PKC, demonstrated that activation of this enzyme leads to increased production of ROM. We used the PKC inhibitor BIM to study the relevance of PKC activation for Lp(a)-induced ROM production. Bisindolyl malemides have structural similarities to staurosporine, but display greater selectivity for PKC and appear to inhibit all PKC isozymes with similar potency [32]. BIM acts as competitive inhibitor of the ATP-binding site on PKC. Our data suggest that Lp(a) acts on an enzyme-
(complex) which requires phosphorylation by PKC for regulation of activity. The comparable time course of lipoprotein-induced and basal chemiluminescence suggests that Lp(a) enhances the recruitment of active enzyme units, rather than suppressing their inactivation, in the latter case, a prolonged plateau phase would have been expected.

Increased levels of intracellular cyclic adenosine monophosphate (cAMP) can suppress the burst of ROM generation in inflammatory cells [13,14] and this pathway is also present in glomeruli [15,16]. The diterpene forskolin elevates intracellular cAMP levels via activation of adenylate cyclase in membrane preparations and intact cells [33]. In our study forskolin, but not the inactive component 1,9 diodeoxy-forskolin, inhibited Lp(a)-induced ROM production.

Several studies added to our understanding of the cross-talk between cAMP, PKC and ROM formation in glomeruli: Elevation of intracellular cyclic AMP levels can inhibit PKC activation by two mechanisms. In platelets, neutrophils and lymphocytes, cAMP, probably through cAMP-dependent protein kinase A activation, inhibits PKC pathways at a step proximal to PKC activation [31,34]. In glomeruli, cAMP suppresses PKC effects on ROM production at a step distal of PKC activation by inhibition of the translocation of PKC from the cytosol to the membrane [16].

In conclusion, Lp(a)-induced ROM production in glomerular cells might be a key finding to better understand the pathophysiological consequences of Lp(a) deposits in human glomerular disease. The finding that a PKC and cAMP sensitive pathway promotes lipoprotein induced ROM production might open new therapeutic approaches to block ROM production in renal disease.

References

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