Statins Prevent Oxidized LDL-Induced Injury of Glomerular Podocytes by Activating the Phosphatidylinositol 3-Kinase/AKT-Signaling Pathway

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The injury of podocytes is associated with alterations of the glomerular size-selective barrier to proteins. In this study, oxidized LDL (oxLDL) but not native LDL induced apoptosis in human cultured podocytes and reduced Akt activity and P-Akt/Akt ratio. Moreover, oxLDL-induced redistribution and loss of nephrin, an adhesion molecule specific for the glomerular slit diaphragm. Nephrin reduction was preceded by inhibition of nephrin tyrosine phosphorylation and of its association with p85 phosphatidylinositol 3-kinase (PI3K). Moreover, three different statins, mevastatin, pravastatin, and simvastatin, inhibited in a dose-dependent manner apoptosis and loss of nephrin induced by oxLDL by stimulating Akt activity. In addition, simvastatin significantly increased the expression of nephrin protein and mRNA by podocytes. The protective effects of statins were blocked by treatment of podocytes with two unrelated pharmacologic inhibitors of PI3K, LY294002 and wortmannin, suggesting a role for PI3K, and by mevalonate, indicating dependency on HMG-CoA reductase activity. Statins directly stimulated Akt phosphorylation ad activity. Finally, oxLDL induced a retraction of cultured podocytes and an increase in the albumin diffusion across their monolayer that was inhibited by treatment with statins. In conclusion, statins reduced the oxLDL-induced apoptosis and loss of nephrin in glomerular podocytes. The statin-induced Akt activation may protect from the loss of nephrin by an inhibition of its redistribution and shedding and by a stimulation of its synthesis. These data provide a rationale for the anti-proteinuric effect of statins.


Podocytes play a central role in the glomerular size-selective barrier to proteins (1). Several studies on different slit diaphragm-associated proteins, such as nephrin, CD2-associated protein, podocin, and α-actinin-4 have outlined their relative contribution in maintaining glomerular size-selective permeability (2). In particular, the critical role of nephrin has emerged. Mutations in the nephrin gene have been found in patients with congenital nephrotic syndrome of the Finnish type, as well as in other patients with nephrotic syndrome (3,4). Moreover, a correlation between changes in nephrin expression and proteinuria has been shown in several experimental models of glomerulonephritis (5,6). We and others have recently shown a reduced expression of nephrin in patients with primary acquired nephrotic syndrome, independently from the initial pathogenic mechanism (7–9). Abnormalities in lipid and lipoprotein metabolism are often observed in patients with nephrotic syndrome and chronic renal diseases (10). The deposition of atherogenic lipoproteins has been implicated in the progression of glomerular injury (11). In particular, oxidized LDL (oxLDL) (1) plays a major role in the pathogenesis of atherosclerosis and in the development of various glomerular diseases (12,13). It has been shown that oxLDL may affect the behavior of endothelial, mesangial, and tubular epithelial cells (14–17). No data are at presently available on a direct effect of oxLDL on podocytes. The first aim of this study was therefore to investigate the effect of oxLDL on human podocyte functions, such as retraction and apoptosis, and on nephrin expression, signaling, and activation of the AKT-dependent survival pathway.

Recently, it has been shown that statins may reduce the urinary excretion of proteins and the urinary loss of podocytes (18,19), a marker of severity of glomerular injury and a predictor of progression to glomerular sclerosis (20). Statins reduce cholesterol synthesis through inhibition of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase and are widely prescribed to hyperlipidemic patients to reduce their risk of atherosclerotic complications. However, statins have additional benefits on vascular function above and beyond their lipid-lowering effects (21).

We therefore aimed to evaluate whether statins may reverse the response of podocytes to oxLDL and whether this may depend on the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway.
Materials and Methods
Reagents
DMEM, BSA fraction V (tested for not more than 1 ng endotoxin per mg), LDL, wortmannin, LY294002 hydrochloride, mevalonate, and all of the secondary antibodies were purchased from Sigma Chemical Company (St Louis, MO). For oxidation, LDL (5 mg/ml) were mixed with 5 μmol CuSO₄, incubated for 18 h at 37°C, and oxidation was evaluated as described previously (22). FCS was from Euroclone Ltd (Wetherby, West Yorkshire, UK). The polyclonal sheep antibody against human Akt and the polyclonal rabbit antisera anti-p85 subunit of PI3-K were from Upstate Biotechnology (Lake Placid, NY). Mouse monoclonal anti-phosphorylated Akt (P-Akt) was from Cell Signaling Technology (Beverly, MA). Mouse monoclonal anti-β-actin and the appropriate isotypic control antibodies were purchased by Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-phosphotyrosine RC20 antibody (anti-P-Tyr) was from Transduction Laboratories (Lexington, KY).

Pravastatin and carboxylate active forms of simvastatin and mevastatin were obtained from Calbiochem (Darmstadt, Germany). Polyclonal anti-nephrin guinea pig antibodies to the extracellular fibronectin domain (GP-N1) and to the intracellular domain (GP-N2) were purchased from Progen Biotechnic (Heidelberg, Germany). An anti-nephrin IgG, monoclonal antibody 48E11 specific for the extracellular fibronectin type III-like motif of the recombinant human nephrin (23) and an IgG1 monoclonal antibody 3D11 specific for the complete intracellular nephrin domain were kindly provided by K. Tryggvason (23). An irrelevant IgG1 isotypic control antibody was purchased from Cedarlane (Hornby, ON, Canada). Pravastatin and carboxylate active forms of simvastatin and mevastatin were obtained from Calbiochem (Darmstadt, Germany).

Culture of Human Podocytes
Primary cultures of human podocytes were established and lines of differentiated podocytes were obtained by infection with a hybrid Adeno5/SV40 virus. Podocytes were characterized and cultured in DMEM containing 10% FCS as described previously (24).
Figure 2. Podocytes expression of nephrin by immunogold scanning electron microscopy. (A and B) Representative micrographs of nephrin expression. Nephrin is detectable by 48E11 monoclonal antibody on the cell surface with a prominent distribution to the periphery (A) and at cell-to-cell contacts (B; black arrows). (C) Representative micrograph of immunogold electronmicroscopy of podocytes performed with an irrelevant isotypic control antibody. N = nucleus. Magnification, ×2000 in A to C.

Immunofluorescence Studies
Immunofluorescence on cultured podocytes was performed as described previously (9). Cells, fixed in 3.5% paraformaldehyde containing 2% sucrose for 15 min at 4°C, were incubated either with the anti-nephrin monoclonal antibody (10 μg/ml) or the irrelevant IgG1 isotypic control, followed by FITC-conjugated anti-mouse IgG. Nephrin expression on cultured podocytes was analyzed semiquantitatively by measuring fluorescence intensity by digital image analysis (Windows MicroImage; CASTI Imaging, Venice, Italy) on images obtained using a low-light video camera on ×400 microscopic fields as described previously (7,9).

Quantitative Real-Time PCR
Quantitative reverse-transcription PCR was performed as described previously (25). Relative quantification by real-time PCR was performed using SYBR-green detection of PCR products in real-time using the iCycler from Biorad (Hercules, MA). Sequence-specific oligonucleotide primers (purchased from MWG-Biotech AG, Ebersberg, Germany) were previously described (26): human nephrin: forward, 5'-AGGACCGAGTCCAGGAACGAAT-3'; reverse, 5'-CTTGAAACCTCGGGAATAAGACA-3'; hGAPDH: forward, 5'-TGGTCACAGGGCTGCTT-3'; reverse, 5'-AGCTTCCCTTCAGCCTT-3'. IQ SYBR Green Supermix was purchased from Biorad. Thermal cycling conditions were as follows: Activation of iTaq DNA polymerase at 95°C for 3 min, followed by 50 cycles of amplification at 95°C for 30 s, 61°C (for nephrin) or 60°C (for GAPDH) for 30 s, and 72°C for 30 s. To detect the log phase of amplification, the fluorescence level (quantification of product) was determined at each cycle. The cycle at which the fluorescence reached threshold was recorded, averaged between triplicates, and normalized to the averaged cycle of threshold value which the fluorescence reached threshold was recorded, averaged between triplicates, and normalized to the averaged cycle of threshold value for GAPDH. Fold change in expression with respect to control (unstimulated cells) was calculated for all samples.

Electron Microscopy
Immunogold labeling was performed on 2.5% paraformaldehyde-fixed cells (27) using a primary-specific antibody or an isotype-matched Ig (Dako) and, as secondary antibody, the 5-nm gold-conjugated rabbit anti-mouse antibody (BBInternational, Cardiff, UK) followed by silver enhancement (silver enhancing kit; BBInternational). Samples were postfixed in 2.5% glutaraldehyde, dehydrated in alcohol, dried, and coated with gold by sputter coating. The specimens were examined in a scanning Jeol T300 electron microscope. Images were obtained via secondary electron at a working distance of 15 to 25 mm and at an accelerating voltage of 20 to 25 kV.

Assessment of Apoptosis
After serum withdrawal, podocytes were treated with oxLDL or native LDL for 24 h in the presence or absence of statins and apoptosis was evaluated using the TUNEL assay analysis (ApopTag Oncor, Gaithersburg, MD).

Akt Kinase Assay
To assay for Akt kinase activity, cells were serum-starved, submitted to different experimental conditions, washed twice in cold PBS, and lysed in ice with 900 μl of lysis buffer containing 1% Triton X-100, 10% glycerol, 137 mmol NaCl, 20 mmol Tris-HCl (pH 7.5), 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mmol phenylmethylsulfonyl fluoride (PMSF), 20 mmol NaF, 1 mmol Na2PO4 and 1 mmol Na3VO4. Equal amounts of lysates (50 μg) were precleared by centrifugation and pre-absorbed with protein A-protein G (1:1) agarose slurry. Immunoprecipitation was performed for 18 h using the immobilized anti-Akt1 IgG1 monoclonal antibody (Cell Signaling Technology) cross-linked to agarose. Immunoprecipitates were washed three times with lysis buffer and twice with the Akt kinase buffer (20 mmol HEPES [pH 7.4] 10 mmol MgCl2, 10 mmol MnCl2), kinase assays were performed for 30 min at 30°C under continuous agitation in kinase buffer containing 200 μmol ATP, 1 μg GSK-3 fusion protein, according to the manufacturer’s instructions of nonradioactive Akt kinase assay (Cell Signaling Technology) as described previously (28).

Immunoprecipitation and Western Blot Analysis
Podocytes were lysed at 4°C for 1 h in a lysis buffer (50 mmol Tris-HCl, pH 8.3, containing 1% Triton X-100, 1 mmol PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 2 mmol sodium orthovanadate) and centrifuged at 15,000 × g. For the detection of nephrin, which is contained within lipid raft microdomains, the lysis buffer was supplemented with 20 mmol CHAPS 3-[3-cholamidopropyl)-(dimethylamo)-no]-1-propanesulfonate (Sigma). Immunoprecipitation with an anti-nephrin IgG monoclonal antibody cross-linked to sepharose-protein A was performed as described (28). Electrophoresis of the immunoprecipitates or of the cell lysates (30 μg of protein per lane) and Western blot analysis were performed as described previously (28).

Albumin Diffusion across Podocyte Monolayer
Podocytes (2 × 105 cells/well) were grown in DMEM supplemented with 10% FCS on 3.0-μm pore size transwell in a 24-well tissue culture plate (Greiner Bio-One, Frickenhausen, Germany) to form a confluent monolayer. Cells were stimulated with 100 μg/ml of oxLDL with or without treatment with various doses of statins for 1 hour at 37°C. The percentage of albumin diffusion was evaluated by a calorimetric assay based on the diffusion of a trypan blue–albumin complex, prepared as follows: Trypan blue (0.36 g) and BSA (0.8 g) were dissolved in 100 ml HBSS and dialyzed for 24 h at 4°C to yield a stable complex with absorption at 590 nm. HPLC using Superdex 200 HR 10/30 (size exclusion; 1 cm × 23.56 cm column) in isocratic water elution condition (flow 0.7 ml/min) of trypan blue–albumin complexes treated or not with oxLDL 100 μg/ml or with 0.1 μmol simvastatin was performed. The results showed overlapping chromatograms, indicating that oxLDL or statins do not influence the size of the complex (data not shown). After plate washings with PBS, 500 μl of trypan blue–albumin complex were added to upper wells, whereas 500 μl of phenol red-free RPMI 1640 were added to bottom wells. Plates were incubated for 30 min at 37°C in continuous agitation, then aliquots of medium from the
bottom wells were transferred to a 96-well plate and absorption at 590 nm was evaluated. Percentage of diffusion was expressed as:

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\% \text{ of diffusion} = \frac{\text{mean Abs}_{590 \text{ nm sample}} - \text{mean Abs}_{590 \text{ nm control}}}{\text{mean Abs}_{590 \text{ nm control}}} \times 100
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**Statistical Analyses**

Data are presented as means ± SD. Statistical and significant differences were determined using ANOVA with the Newmann-Keuls or Dunnet multicomparison tests when appropriate.

**Results**

**Effect of oxLDL on Podocyte Apoptosis, Akt Activation, and Nephrin Expression**

Oxidatively modified LDL, but not native LDL, induced apoptosis of podocytes in a dose-dependent manner (Figure 1A). To test whether oxLDL influences Akt-dependent survival pathway, podocytes were incubated for 1 h with increasing concentrations of oxLDL. As seen in Figure 1B, oxLDL induced a dose-dependent reduction of the basal Akt activity. This was associated with a dose-dependent dephosphorylation of Akt as indicated by the reduction of P-Akt/Akt ratio (Figure 1, C and D). In contrast, native LDL did not affect P-Akt/Akt ratio.

Nephrin was detectable on cultured podocytes by scanning immunogold electron microscopy on the peripheral cell surface and at cell-to-cell junctions (Figure 2, A and B). By immunofluorescence microscopy on confluent monolayers of nonpermeabilized cells, nephrin was mainly detectable on the peripheral cell surface (Figure 3A). OxLDL induced a reduction of nephrin expression in respect to control (Figure 3). The loss of nephrin
was detectable after 30 min of incubation (Figure 3B) and peaked at 1 h (Figure 3D). As seen in Figure 3C, a redistribution of nephrin was observed with aspects of capping. The reduction of nephrin expression was already evident using a dose of 1 μg/ml oxLDL and increased in a dose-dependent manner (Figure 3J). Nephrin reduction was also observed after 24 h of incubation with a dose as low as 10 μg/ml oxLDL, which induced only minimal apoptosis (Figure 3, E and J). Native LDL induced only a slight reduction of nephrin expression (Figure 3, F and J). The rapid redistribution of nephrin was associated with changes in the cytoskeleton of podocytes, characterized by a reduction of stress fibers and by peripheral accumulation of F-actin in respect to control (Figure 3, G and H). Treatment with cytochalasin-B, a compound that affects the microfilaments of the microtubular system and the surface redistribution of nephrin (8), inhibited the loss of nephrin, which assumed a more granular immunofluorescence pattern on the cell surface (Figure 3I). The nephrin reduction was confirmed by Western blot analysis of cell lysates (Figure 4, A and B). In addition, nephrin reduction in the cell lysates was inhibited by cytochalasin-B and by the protease inhibitors PMSF and cystatin (Figure 4, A and B). OxLDL also inhibited in a dose-dependent manner the tyrosine phosphorylation of nephrin and the association of p85 PI3K with nephrin (Figure 4C), which has been previously shown to be a critical step in nephrin-induced signaling (29). This event was observed at 15 min and preceded the loss of nephrin protein expression.

Statins Inhibit the Effect of oxLDL on Podocyte Apoptosis, Akt Activation, and Nephrin Expression

Exposure to HMG-CoA reductase inhibitors, statins, inhibited apoptosis of podocytes induced by 100 μg/ml oxLDL. The effect of statins was dose-dependent and no significant difference was observed among the three statins used (Figure 5A). Statins alone at the low doses (0.01 to 1 μmol) used did not affect apoptosis of podocytes. However, at a high concentration (50 μmol), simvastatin, which is lipophilic, induced apoptosis (25.7 ± 5.3% at 24 h), whereas pravastatin, which is hydrophilic, did not (2.2 ± 0.7%). Incubation of podocytes with mevalonate reversed the protection of statins on oxLDL-induced apoptosis (data not shown). Statins are known to activate the Akt/PI3K survival pathway (30). Treatment of podocytes with two unrelated PI3K pharmacologic inhibitors, wortmannin (0.1 μmol) and LY294002 (10 μmol) abrogated the anti-apoptotic effect of statins (Figure 5B), suggesting that this effect was at least in part dependent on the activation of PI3K. Wortmannin and LY294002 alone induced apoptosis in cells deprived of serum by inhibiting the basal PI3K/Akt survival pathway (Figure 5B). The evidence that simvastatin did not abrogate this apoptotic effect suggests that the survival effect of statins is not dependent on the activation of pathways different from PI3K/Akt (Figure 5B). Moreover, statins also exerted a significant inhibition of apoptosis induced by vincristine (Figure 5C). The treatment of podocytes with the three statins also prevented the reduction of P-Akt/Akt ratio induced by oxLDL (Figure 6, A and B). The protective effect of statins on Akt phosphorylation was prevented by LY294002 (Figure 6, A and B). To assess whether statins may directly stimulate Akt phosphorylation and activity, podocytes were deprived of serum overnight, a condition that inhibits the basal Akt phosphorylation, and then incubated with 0.1 μmol statins alone for 1 h at
Statins were able to induce an increase in P-Akt/Akt ratio, significantly inhibited by LY294002, and in Akt activity (Figure 6, C, D, and E).

Moreover, we observed that statins prevented the loss of nephrin expression induced by oxLDL both at 1 h (Figure 7, A and C to G) and at 24 h (Figure 7, B, H, and I). At 1 h, no significant variation in nephrin expression was observed after incubation with statins alone (Figure 7A). In contrast, after 24-h incubation, statins significantly enhanced nephrin expression by podocytes (Figure 7B). The protective effect of statins on the reduced expression of nephrin induced by oxLDL was also observed by Western blot analysis (Figure 7J). In addition, statins restored the association of p85 PI3K with tyrosine phosphorylated nephrin that was inhibited by oxLDL (Figure 7K). The effect of statins on nephrin expression was dependent on HMG-CoA reductase, because it was inhibited by mevalonate (Figure 8A), and on Akt/PI3K pathway, because it was blocked by LY294002 and wortmannin (Figure 8A). By quantitative reverse-transcriptase PCR, we observed a slight but significant reduction of nephrin mRNA expression after stimulation with oxLDL (Figure 8B). Simvastatin not only antagonized the reduction of nephrin mRNA expression induced by oxLDL but also significantly increased the expression of nephrin mRNA by podocytes (Figure 8B), suggesting a direct effect on nephrin gene transcription. This was concomitant with the significant increase in nephrin protein expression (Figure 8A). This effect was dependent on PI3K activation because it was inhibited by LY294002 and on HMG-CoA reductase activity, because it was inhibited by mevalonate (Figure 8B).

These observations suggest that the Akt activation seen in response to the treatment of podocytes with statins provides protection against oxLDL-mediated injury.

**Statins Inhibit the oxLDL-Induced Effects on Podocyte Barrier Function**

Podocytes regulate protein diffusion through the glomerular barrier. We investigated the effect of oxLDL on podocyte function by studying albumin diffusion across podocyte monolayers. OxLDL induced a dose-dependent increase in albumin diffusion (Figure 9A). Statins were able to inhibit in a dose-dependent manner the permeability to albumin observed after stimulation with oxLDL (Figure 9B). In particular, simvastatin was more effective in inhibiting albumin diffusion. This protective effect was dependent on PI3K activation because it was inhibited by LY294002 and on HMG-CoA reductase activity, because it was inhibited by mevalonate (Figure 8B). By scanning electron microscopy, oxLDL induced cell retraction and gap formation in the podocyte monolayer, suggesting an inter-
cellular diffusion of albumin caused by cell retraction (Figure 10, A and B). This was associated with a redistribution of cell cytoskeleton (Figure 10, D and E). We can not exclude that detachment of a few cells may account for the increased permeability of podocyte monolayer; however, no changes in cell viability were observed by XTT reduction-based assay after 1-h incubation with oxLDL. Statins prevented the retraction of podocytes and the cytoskeletal alterations induced by oxLDL (Figure 10, C and F), thus maintaining the integrity of podocyte monolayers.

Discussion
In our study, we demonstrated that the treatment with statins reduced in a dose-dependent manner the oxLDL-induced apoptosis and loss of nephrin in glomerular podocytes by activating the PI3K/AKT-dependent pathway.

OxLDL has been observed to induce abnormalities in endothelial function, which may be relevant for the progression of atherosclerotic lesions (14). OxLDL inhibits the expression of constitutive endothelial nitric oxide synthase, stimulates the apoptosis of endothelial cells, induces expression of adhesion molecules on endothelial cells, and facilitates inflammatory cells to adhere to the intima of vessels (31–33). The apoptotic effect of oxLDL on endothelial cells could be attributed to oxidation products of phosphatidylcholine or to oxysterols (32).

In our study, we observed that oxLDL induced apoptosis also of cultured human podocytes. Glomerular podocytes are highly specialized cells with a critical role in the formation of the glomerular filtration barrier (1,2). Recently, the critical role of nephrin, in maintaining glomerular permeability, has stressed the role of podocytes (3,4). Nephrin is a transmem-

Figure 6. Statins inhibit dephosphorylation of Akt induced by oxLDL and directly stimulate Akt phosphorylation and activity. (A) Densitometric analysis of P-Akt/Akt ratio. Data are expressed as mean ± SD of three different experiments. ANOVA with Newmann-Keuls multicomparison test was performed: all experimental conditions versus vehicle alone (*P < 0.05); oxLDL plus statins versus oxLDL (⁎P < 0.05); oxLDL plus statins plus LY294002 versus oxLDL plus statins (⁎⁎P < 0.05). (B) Immunoblots of a representative experiment. (C and D) To evaluate whether statins stimulate phosphorylation of Akt, podocytes were serum-starved overnight and then stimulated for 1 h at 37°C with 0.1 μmol mevastatin (M), pravastatin (P), or simvastatin (S) added to cells immediately before stimulation and, to inhibit PI3K, in the presence of LY294002 (10 μmol) to inhibit PI3K. Podocyte lysates were immunoblotted with anti-P-Akt, anti-Akt, or anti-β-actin antibodies. (E) Densitometric analysis of P-Akt/Akt ratio. Data are expressed as mean ± SD of three different experiments. ANOVA with Newmann-Keuls multicomparison test was performed: *P < 0.05 statins versus vehicle alone; †P < 0.05 statins plus LY294002 versus statins alone. (D) Immunoblots of a representative experiment. (E) To evaluate whether statins stimulate Akt activity, podocytes were serum-starved overnight and then stimulated for 1 h at 37°C with increasing doses of simvastatin (S). Akt activity was measured using recombinant GSK-3α/β as substrate for phosphorylation. Data are expressed as average intensity of densitometric analysis (mean ± SD of three different experiments). ANOVA with Dunnett multicomparison test was performed versus vehicle alone (*P < 0.05).
brane protein of the Ig superfamily, specifically expressed in the slit diaphragm (34–38). It has been suggested that, in a fashion similar to other cell adhesion molecules, nephrin might function in intracellular signal transduction (39,40).

In this study, we observed that oxLDL induced loss of nephrin expression from cultured podocytes. Moreover, the loss of nephrin was preceded by the inhibition of its tyrosine phosphorylation and of its association with p85 PI3K. It has been recently shown that nephrin once phosphorylated associates with PI3K and stimulates the AKT-dependent signaling (29). This pathway, which plays a critical role in the remodeling of actin cytoskeleton, in the control of protein trafficking, and in the cell survival (41), may have a role in nephrin-dependent cytoskeleton activation. The loss of nephrin induced by oxLDL...
Figure 8. PI3K-dependent effect of statins on nephrin protein and mRNA expression. (A) Semiquantitative analysis of nephrin expression evaluated as relative fluorescence intensity after cell stimulation for 24 h at 37°C with 10 μg/ml oxLDL or with 10 μg/ml oxLDL plus 0.1 μmol simvastatin (S) or with 0.1 μmol of S alone in the absence or presence of two PI3K pharmacologic inhibitors, 10 μmol LY294002 (LY), 0.1 μmol wortmannin (W), or 200 μmol mevalonate (Mev). Data are expressed as mean ± 1 SD of three different experiments. ANOVA with Newmann-Keuls multicomparison test was performed: *P < 0.05 oxLDL, oxLDL plus S plus LY, oxLDL plus S plus W, or Mev, and S alone versus vehicle; §P < 0.05 oxLDL plus S plus LY, W, or Mev versus oxLDL plus S. (B) Nephrin mRNA expression analyzed by quantitative real-time PCR in unstimulated podocytes or podocytes stimulated with 0.1 μmol simvastatin (S), with 10 μg/ml oxLDL or with 10 μg/ml oxLDL plus 0.1 μmol simvastatin (S) in the absence or presence of 10 μmol LY294002 (LY) or 200 μmol mevalonate (Mev) for 16 h. The normalized expression of nephrin gene with respect to GAPDH was computed for all samples. Values are expressed as fold changes with respect to control and are the mean ± 1SD of three independent experiments performed in triplicate. ANOVA with Newmann-Keuls multicomparison test was performed: *P < 0.05 S or oxLDL or oxLDL plus S versus vehicle; #P < 0.05 oxLDL plus S versus oxLDL; §P < 0.05 oxLDL plus S plus LY or oxLDL plus S plus Mev versus oxLDL plus S.

Figure 9. Effect of oxLDL and statins on podocyte barrier function. (A) Trypan blue–albumin complex diffusion across a monolayer of podocytes treated with increasing doses of oxLDL. (B) Percentage of diffusion across a monolayer of podocytes stimulated with 100 μg/ml of oxLDL with or without treatment with various doses of mevastatin (M), pravastatin (P), or simvastatin (S) or statins alone (1 μmol) for 1 h at 37°C. Data are expressed as percentage of albumin diffusion as indicated in Materials and Methods. Values are the mean ± 1SD of four experiments performed in duplicate. ANOVA with Newmann-Keuls multicomparison test was performed: all experimental conditions versus vehicle alone (*P < 0.05); oxLDL plus statins (P, M, and S) at different concentrations (0.01 or 0.1 or 1 μmol) versus oxLDL (*P < 0.05). (C) Percentage of diffusion across a monolayer of podocytes treated for 1 h with oxLDL (100 μg/ml) or oxLDL plus simvastatin (0.1 μmol S) or oxLDL plus S plus LY294002 or Mevalonate or LY294002 and Mevalonate and simvastatin alone. Data are expressed as mean ± 1 SD of three different experiments. ANOVA with Newmann-Keuls multicomparison test was performed: *P < 0.05 oxLDL versus vehicle; *P < 0.05 oxLDL plus S versus oxLDL; §P < 0.05 oxLDL plus S plus LY294002 and oxLDL plus S plus Mevalonate versus oxLDL plus S.
could not be ascribed to a reduced synthesis, because it occurred within 1 h, but rather to a nephrin shedding from the surface and to its catabolism. We previously observed that several stimuli acting on cell cytoskeleton can induce nephrin shedding from the surface of podocytes (8). The observation that the loss of nephrin was reduced by treatment with cytochalasin B, which interferes with the cytoskeleton, and by antiproteases suggests that the redistribution of nephrin is caused by cytoskeleton activation and that its shedding depends on the activation of cell proteases. As a functional result, oxLDL induced a retraction of cultured podocytes and an increase in the albumin diffusion across their monolayer. These data indicate a direct effect of oxLDL on glomerular podocyte function that may be relevant for the progression of glomerulosclerosis. In addition, we observed that oxLDL inhibits Akt activation favoring dephosphorylation of Akt in podocytes, suggesting an interference of oxLDL with the pathway involved in the activation of Akt. Several studies have shown that Akt is the major effector of PI3K survival signaling (42–43), thus apoptosis induced by oxLDL may depend on the inhibition of this pathway.

In our study, we demonstrated that statins inhibited, at low doses, apoptosis and loss of nephrin induced by oxLDL, as well as the reduced P-Akt/Akt ratio. We observed that the protective effects of statins were blocked by treatment with two unrelated pharmacologic inhibitors of PI3K, wortmannin and LY294002, suggesting the role of PI3K. PI3K activation catalyzes phosphorylation of phosphatidylinositol, leading to Akt binding. The binding of Akt to 3'-phosphatidylinositol phosphates results in its translocation from cytosol to plasma membrane and phosphorylation of threonine 308 and serine 473 residues (44).

Recent studies have revealed a link between statins and Akt (30). Statins have been shown to rapidly promote the activation of Akt in endothelial cells (30). We observed that statins prevented the early dephosphorylation of Akt and the reduced association of PI3K with the phosphorylated nephrin induced by oxLDL. Moreover, we found that statins may directly stimulate Akt activity and phosphorylation. In addition, simvastatin was found to markedly enhance nephrin gene transcription and protein expression, effects that were inhibited by LY294002. Therefore, statin-induced Akt activation may be responsible for two different mechanisms contributing to protect from the loss of nephrin: An early effect on inhibition of its redistribution and shedding and a delayed effect on stimulation of its synthesis. Finally, statins inhibited the permeability to albumin and the cell retraction induced by oxLDL. These results are consistent with the role of statins in vascular protection (45).

In conclusion, the results of this study indicate that glomerular podocytes exposed to oxLDL undergo apoptosis and loss of nephrin, a key component of the glomerular filtration barrier. These events were inhibited by statins through an upregulation of the Akt/PI3K signaling pathway, providing a rationale for the observed anti-proteinuric effect of statins (18,19).

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