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Oxidative Stress-mediated Mesangial Cell Proliferation Requires RAC-1/Reactive Oxygen Species Production and β4 Integrin Expression*

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Lipid abnormalities and oxidative stress, by stimulating mesangial cell (MC) proliferation, can contribute to the development of diabetes-associated renal disease. In this study we investigated the molecular events elicited by oxidized low density lipoproteins (ox-LDL) in MC. We demonstrate that in MC cultured in the presence of ox-LDL, survival and mitogenic signals on Akt and Erk1/2 MAPK pathways are induced, respectively. Moreover, as shown by the expression of the dominant negative Rac-1 construct, we first report that ox-LDL-mediated cell survival and cell cycle progression depend on Rac-1 GTPase-mediated reactive oxygen species production and on epidermal growth factor receptor transactivation. By silencing Akt and blocking Erk1/2 MAPK pathways, we also demonstrate that these signals are downstream to Rac-1/reactive oxygen species production and epidermal growth factor receptor activation. Finally, by endogenous depletion of β4 integrin, expressed in MC, we provide evidence that the expression of this adhesion molecule is essential for ox-LDL-mediated MC dysfunction. Our data identify a novel signaling pathway involved in oxidative stress-induced diabetes-associated renal disease and provide the rationale for therapeutically targeting β4 integrin.

Abnormalities in lipoprotein metabolism are commonly observed in patients with renal disease. Specifically, hyperlipidemia and glomerular lipid deposition of atherogenic lipoproteins (low density lipoproteins (LDL),[2] and their oxidized variants, ox-LDL) are implicated in key pathobiological processes involved in the development of glomerular diseases, including mesangial cell (MC) hypercellularity (1). The relevance of mitogen intracellular signaling in mesangial proliferative disease has only recently been recognized (2). Indeed, accumulation of atherogenic lipoproteins within the glomerulus, by activating membrane receptor protein-tyrosine kinases (RPTK), such as the epidermal growth factor receptor (EGFR), triggers MAPK cascades leading to cyclin/cyclin-dependent kinase activation of DNA synthesis and MC proliferation (2). Moreover, in smooth muscle cells, ox-LDL (3) has been shown to trigger the phosphatidylinositol 3-kinase/Akt signaling pathway (4). These data thus suggest that atherogenic lipoproteins may act as one of the major endogenous modulators for mitogenic signaling response within the glomerulus.

Although superoxide anions and hydrogen peroxide are generally considered to be toxic, recent evidence suggests that the production of the reactive oxygen species (ROS) might be an integral component of membrane receptor signaling (5). In mammalian cells, a vast array of intracellular stimuli have been shown to induce a transient increase in the intracellular ROS concentrations, and specific inhibition of ROS generation results in a complete blockage of stimulant-dependent signaling (5). In particular, growth factor-mediated ROS generation seems to be necessary for propagation of downstream mitogenic and antiapoptotic signals (5).

Rho family GTPases are implicated in the regulation of different cell functions, including motogenesis and mitogenesis, and orchestrate gene expression to mitogenic cues from both soluble factors and extracellular matrix proteins (6). The family members, Rac-1 and Rac-2, participate in the regulation of ROS generation in several cell types (7–9). However, despite a clear association between Rac-1 and ROS generation, the ROS-signaling targets are still poorly defined.

Proliferation obeys a bimodal control by integrins and RPTKs, which together prime mitogenic signals in response to cell adhesion and growth factor, respectively (10). Strong evidence indicates that integrins and RPTK-dependent signals need to be integrated at various levels to cause cell proliferation (10, 11). Co-immunoprecipitation studies indicate that many of the Shc-linked integrins form a complex with RPTKs. In particular, α5β1 combines with EGFR, whereas α6β4 combines with EGFR, the related RPTK Erb-B2, and the hepatocyte growth factor receptor Met (12–16).

The α6β4 integrin is a laminin-5 receptor expressed in many epithelial cells, in endothelial cells, and in MC (17–19). β4 integrin, originally identified as a tumor-associated antigen (20), was subsequently documented in human premalignant lesions

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[2] The abbreviations used are: LDL, low density lipoprotein; n-LDL, nonmodified LDL; ROS, reactive oxygen species; DPI, diphenylene iodonium; NAC, *N*-acetylcysteine; MC, mesangial cell; ox-LDL, oxidized low density lipoprotein; EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; shRNA, short hairpin RNA; FACS, fluorescence-activated cell sorter; AGE, advanced glycated end product; CHX, cycloheximide; siRNA, short interfering RNA; mAb, monoclonal antibody.

TABLE 1

Effect of ox-LDL on MC cell cycle progression

Serum-starved MC were stimulated for the indicated times with ox-LDL (50 mg/ml), 10% fetal calf serum (FCS), AGE (1.2 mg/ml), or n-LDL (50 μg/ml). After stimulation, the cells were fixed with ethanol, and DNA content was evaluated by flow cytometry. The percentage of cells in each phase of the cell cycle was determined by ModFit LT software (Verity Software House Inc.). The numbers are the mean ± S.D. of three independent experiments each performed in duplicate.

Cell cycle phases	Percentage of cells									
	48 h					72 h				
	Control	Ox-LDL	FCS 10%	AGE	n-LDL	Control	Ox-LDL	FCS 10%	AGE	n-LDL
G_0/G_1	56 ± 4	47 ± 3	45 ± 3	63 ± 5	55 ± 3	58 ± 4	40 ± 3	41 ± 3	68 ± 5	57 ± 4
S	36 ± 3	45 ± 4[a]	46 ± 3[a]	33 ± 3[b]	37 ± 2	32 ± 3	48 ± 5[a]	50 ± 4[a]	28 ± 3[b]	34 ± 3
G_2/M	8 ± 1	8 ± 1	9 ± 2	4 ± 2	8 ± 2	10 ± 1	12 ± 1	9 ± 2	4 ± 2	9 ± 2

[a] $p < 0.05$, ox-LDL treatment or 10% fetal calf serum *versus* control is indicated.
[b] $p < 0.05$ with AGE treatment *versus* control is indicated.

and incipient neoplasms (11, 21). Data indicate that integrins such as β4 can trigger, independently of growth factor stimulation, intracellular signals leading to the activation of the Erk1/Erk2 MAPK, Shc-Ras-phosphatidylinositol 3-kinase, and Rac-1 signaling pathways (22, 23).

The aim of this study was to investigate the molecular mechanisms associated with hypercellularity of MC exposed to ox-LDL. Here we demonstrate that the Akt and the Erk1/2 MAPK signaling pathways are downstream events of ox-LDL-mediated Rac-1/ROS/EGFR-dependent cell survival and cell cycle progression, respectively. Moreover, by silencing β4 integrin we also provide evidence that β4 integrin acts upstream of the Rac-1/ROS-mediated pathway and cooperates with the EGFR to control ox-LDL-mediated MC cycle progression.

EXPERIMENTAL PROCEDURES

Reagents—RPMI medium, bovine serum albumin, glycated human albumin, protein A-Sepharose, NAC, diphenylene iodonium (DPI), an NADPH oxidase inhibitor, propidium iodide, and cycloheximide were all from Sigma. Bovine calf serum (endotoxin-tested) was obtained from HyClone (Logan, UT). Trypsin was purchased from Difco. Nitrocellulose filters, horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG, molecular weight markers, and chemiluminescence reagents (ECL) were from Amersham Biosciences. The EGFR inhibitor, AG1478, the pharmacological inhibitor of Akt, 1L6-Hydroxymethyl-chiro-inositol-2-(*R*)-2-*O*-methyl-3-*O*-octadecyl-*sn*-glycerocarbonate, and Erk1/2 MAPK inhibitor, PD98059, were purchased from Calbiochem. Endotoxin contamination of LDL preparation was tested by the *Limulus* amebocyte assay, and the concentration was <0.1 ng/ml.

Isolation, Characterization, and Oxidation of LDL—Blood from healthy donors was processed according to Redgrave and Carlson (24). Plasma was brought to a density of 1.10 g/ml and processed as described previously (25). The density of the LDL was measured in the tubes where the highest levels of cholesterol were found. LDL was dialyzed against 0.02 M/liter EDTA-free phosphate buffer, pH 7.4, containing 0.15 M/liter NaCl. LDL was adjusted to 0.2 mg/ml protein concentration. $CuSO_4$ was added for 24 h (37 °C) at a ratio of 25 μM/mg LDL protein (n-LDL). Oxidation was stopped by the addition of 1 mM/liter EDTA and 0.02 mM/liter butylated hydroxytoluene (26).

Antisera—Anti-β-actin, anti-p21, anti-cyclin D1, anti-Erk1/2 MAPK, and the extracellular domain of anti-β4 integrin antibodies (H-101 and N-20) were obtained from Santa Cruz Biotechnology, Heidelberg, Germany. Anti-phospho-Akt (Ser-473/Thr-308), anti-Akt, anti-phospho-Erk1/2 MAPK, and anti-phospho-EGFR antisera were from Cell Signaling Technology (Beverly, MA). Anti-Rac-1 was from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-LOX-1 antibody was from R & D Systems. Anti-EGFR and anti-β1 integrin antisera were kindly provided by P. Defilippi. The rat monoclonal antibody (mAb) 439-9B and the mouse mAb 450-11A, raised against the human β4 subunit, were used (20, 27).

Cell Culture and Transfection—MC were cultured as described previously (28). The parental MC were stably transfected, by the Lipofectin method, with the pRC/CMV vector alone or carrying the truncated cytoplasmic domain of the β4 integrin (β4L) acting as dominant negative protein. The parental MC were transiently transfected with a dominant negative mutant of Rac-1 (V12N17Rac) or the empty vector (pEXV) kindly provided by S. Giordano. MCF7 cells were obtained from American Type Culture Collection (Manassas, VA) and maintained as described (27).

Silencing of Endogenous Akt, β1, and β4 Integrins—The inactivation of β4 was obtained by Lipofectamine PLUS method (Invitrogen) using pSUPERretro vector containing β4-shRNA or a scramble RNA sequences as described previously (27, 29). After selection, positive and negative clones were analyzed by Western blot. β4-shRNA MC clone was transiently transfected with the β4 full-length construct (27). To obtain inactivation of Akt and β1 integrin. MC were transiently transfected with purified duplex siRNAs for Akt or β1 integrin and for scramble controls purchased from Qiagen (Valencia, CA). Transfection was performed using Lipofectamine, according to the vendor's instructions. Cell viability was evaluated at the end of the experiment.

Immunoprecipitation and Western Blot Analysis—MC monolayers were serum-starved for 24 h at 37 °C and stimulated with different stimuli, as indicated. Cells were lysed, and protein concentration was obtained as described previously (30). In selected experiments the anti-EGFR pharmacological inhibitor, AG1478 (250 nM/liter), NAC (20 mM/liter), DPI (10 μM/liter), Erk1/2 MAPK inhibitor (35 μM/liter), PD98059, or blocking anti-LOX-1 antibody (1 μg/ml) were used. Proteins (50 μg) were separated on SDS-PAGE transferred to nitrocellulose membranes and analyzed by Western blot. Reaction with antibodies and detection with chemiluminescence system were performed as described previously (30).

Flow Cytometry—To analyze cell cycle progression, MC were fixed with 70% ethanol. DNA was stained with propidium

iodide and analyzed with a flow cytometer (FACScan, Immunocytometry Systems). In selected experiments DPI, AG1478, PD98059, or 1L6-hydroxymethyl-chiro-inositol-2-(*R*)-2-*O*-methyl-3-*O*-octadecyl-*sn*-glycerocarbonate (10 μM/liter) inhibitors and anti-LOX-1 antibody were used. The percentage of cells in each phase of the cell cycle was determined by ModFit LT software (Verity Software House, Inc., Topsham, ME). The fraction of apoptotic cells was quantified by analysis of the sub-G_1 peak (sub-diploid cells). FACS was used for analyzing β4 and β1 integrins expression. Fluorescein isothiocyanate-conjugated anti-rat and anti-mouse IgG (Sigma) were used as secondary antibody.

Detection of ROS—2′,7′-Dichlorofluorescein diacetate (20 mM/liter final concentration) was added to MC in the various cultured conditions. At the times indicated the cells were subjected to FACS analysis and processed as described previously (25). H_2O_2 (100 μM/liter) was used as a positive control.

Assay for Rac1-GTP Loading—MC transfected with the dominant negative mutant of Rac-1 (V12N17Rac) or the corresponding empty vector (pEXV) were serum-deprived for 24 h and untreated or treated with ox-LDL. Cells were lysed as described (31). Lysates were clarified by centrifugation and incubated with recombinant GST-PAK precoupled to Sepharose-glutathione beads (Amersham Biosciences) and then washed three times in lysis buffer (32). The GST-PAK precoupled Sepharose-glutathione beads were used to pull down GTP-bound Rac-1 from cell lysates. The samples were separated by SDS-PAGE and transferred to nitrocellulose filters. The filters were probed with an anti-Rac-1 antibody.

Real-time Quantitative PCR—MC, preincubated or not with cycloheximide (20 μg/ml), were stimulated with ox-LDL (50 μg/ml). mRNA quantification was performed by real-time quantitative PCR, using the ABI PRISM 7700 Sequence detection system and the SYBR Green Master Mix Kit (both from Applied Biosystems). β-Glucuronidase (GUS) gene was used as standard reference. The relative expression of β4 integrin

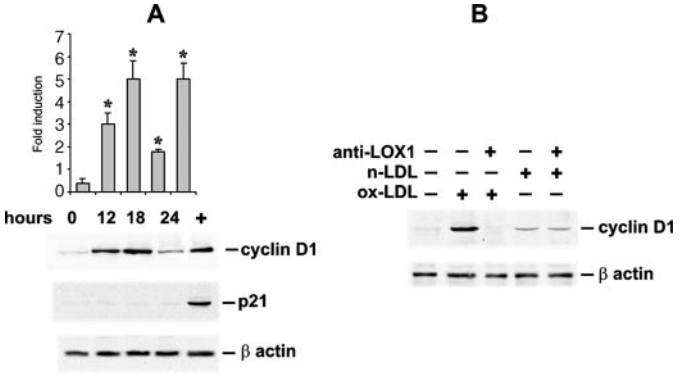

FIGURE 1. **Ox-LDL induce the expression of cyclin D1.** *A,* MC untreated or treated with ox-LDL for indicated time intervals were lysed and subjected to SDS-PAGE. The filter was immunoblotted with an anti-cyclin D1, an anti-p21, and an anti-β-actin antibodies. Fetal calf serum- or AGE-treated MC were used as cyclin D1 or p21 positive control, respectively (+). *B,* cell extracts from MC, stimulated or not with ox-LDL or n-LDL, in the absence or in the presence of a blocking anti-LOX-1 receptor antibody, were analyzed for cyclin D1 and β actin expression by Western blot. Three different experiments were performed with comparable results.

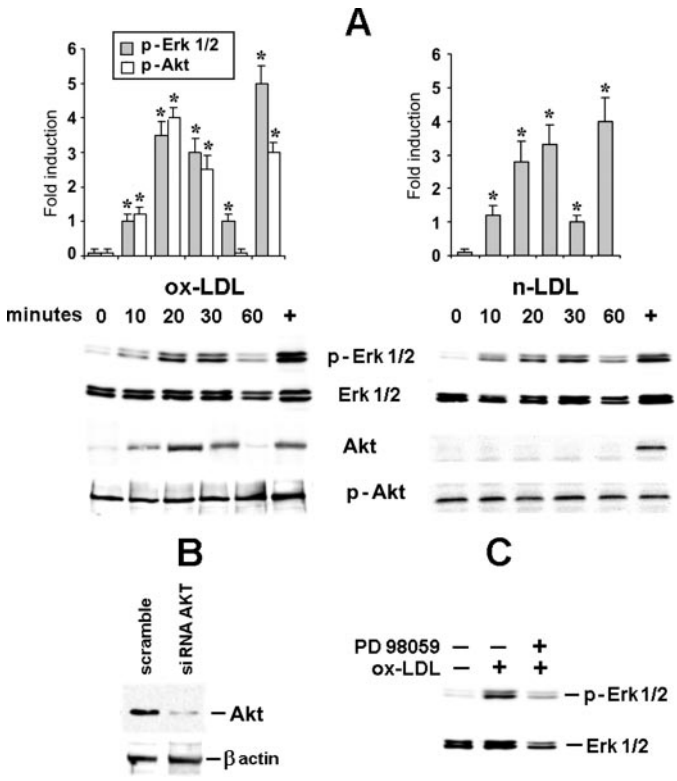

FIGURE 2. **Akt and Erk1/2 MAPK are activated in MC in response to ox-LDL.** *A,* cell extracts from MC stimulated for the indicated time intervals with ox-LDL (50 μg/ml) or n-LDL (50 μg/ml) were analyzed by Western blot with an anti-total and anti-p-Erk1/2 MAPK, or anti-total and anti-p-Akt antibodies, respectively. Interleukin-3-treated endothelial cells were used as positive control (+). *B,* MC transfected with an siRNA Akt or with the scrambled sequence (*scramble*) were lysed. The filter was immunoblotted with an anti-Akt and an anti-β actin antibody. *C,* MC, pretreated or not with PD98059 (35 μM/liter), were stimulated with ox-LDL, lysed, and analyzed for p-Erk1/2 MAPK and Erk1/2 MAPK. Four different experiments were performed with comparable results.

TABLE 2

Effect of LOX-1 blockage or Akt-specific inhibitor on ox-LDL-induced MC cell cycle progression

Serum-starved MC, pretreated or not with a pharmacological inhibitor of Akt (10 μM/liter) or with a blocking anti-LOX-1 antibody, were stimulated for 48 h with ox-LDL (50 μg/ml) or 10% fetal calf serum (FCS). After stimulation, the cells were fixed with ethanol, and DNA content was evaluated by flow cytometry. The percentage of cells in each phase of the cell cycle was determined by ModFit LT software (Verity Software House Inc.). The percentage of subdiploid cells was calculated as indicated under "Experimental Procedures." The numbers are the mean ± S.D. of three independent experiments each performed in duplicate.

Cell cycle phases	Percentage of cells								
	Control			Akt inhibitor			Anti-LOX-1		
	Control	Ox-LDL	FCS 10%	Control	Ox-LDL	FCS 10%	Control	Ox-LDL	FCS 10%
G_0/G_1	58 ± 5	48 ± 3	48 ± 3	55 ± 4	61 ± 3	44 ± 3	59 ± 4	63 ± 3	45 ± 3
S	30 ± 4	46 ± 4	43 ± 3	29 ± 3	28 ± 2[a]	46 ± 3	28 ± 3	27 ± 5[a]	48 ± 4
G_2/M	12 ± 2	6 ± 1	9 ± 2	16 ± 1	11 ± 2	10 ± 2	13 ± 1	10 ± 1	7 ± 2
Apoptosis					17 ± 3				

[a] $p < 0.05$, ox-LDL *versus* ox-LDL + Akt inhibitor or ox-LDL + anti-LOX-1 is indicated.

TABLE 3

Role of Akt and MAPK on ox-LDL-mediated MC cell cycle progression

MC, transiently transfected with a scramble sequence or with an siRNA Akt, or MC pretreated with the Erk1/Erk2 MAPK inhibitor, PD98059 (35 μM/liter), were serum-starved and stimulated for 48 h with ox-LDL (50 μg/ml) or with 10% fetal calf serum (FCS). After stimulation, the cells were fixed with ethanol, and DNA content was evaluated by flow cytometry. The percentage of cells in each phase of the cell cycle was determined by ModFit LT software (Verity Software House Inc.). The percentage of subdiploid cells was calculated as indicated under "Experimental Procedures." The numbers are the mean ± S.D. of three independent experiments each performed in duplicate.

Cell cycle phases	Percentage of cells								
	Scramble			siRNA Akt			PD98059		
	Control	Ox-LDL	FCS 10%	Control	Ox-LDL	FCS 10%	Control	Ox-LDL	FCS 10%
G_0/G_1	61 ± 3	49 ± 3	42 ± 3	60 ± 4	63 ± 3	43 ± 2	55 ± 4	58 ± 4	47 ± 3
S	28 ± 2	43 ± 2	50 ± 3	29 ± 2	25 ± 5[a]	49 ± 4	26 ± 3	30 ± 3[a]	43 ± 3
G_2/M	11 ± 1	8 ± 1	8 ± 2	11 ± 1	12 ± 1	8 ± 2	19 ± 1	12 ± 1	10 ± 2
Apoptosis					20 ± 2				

[a] $p < 0.05$, ox-LDL-treated: scramble expressing cells *versus* siRNA Akt expressing cells or PD98059-treated cells are indicated.

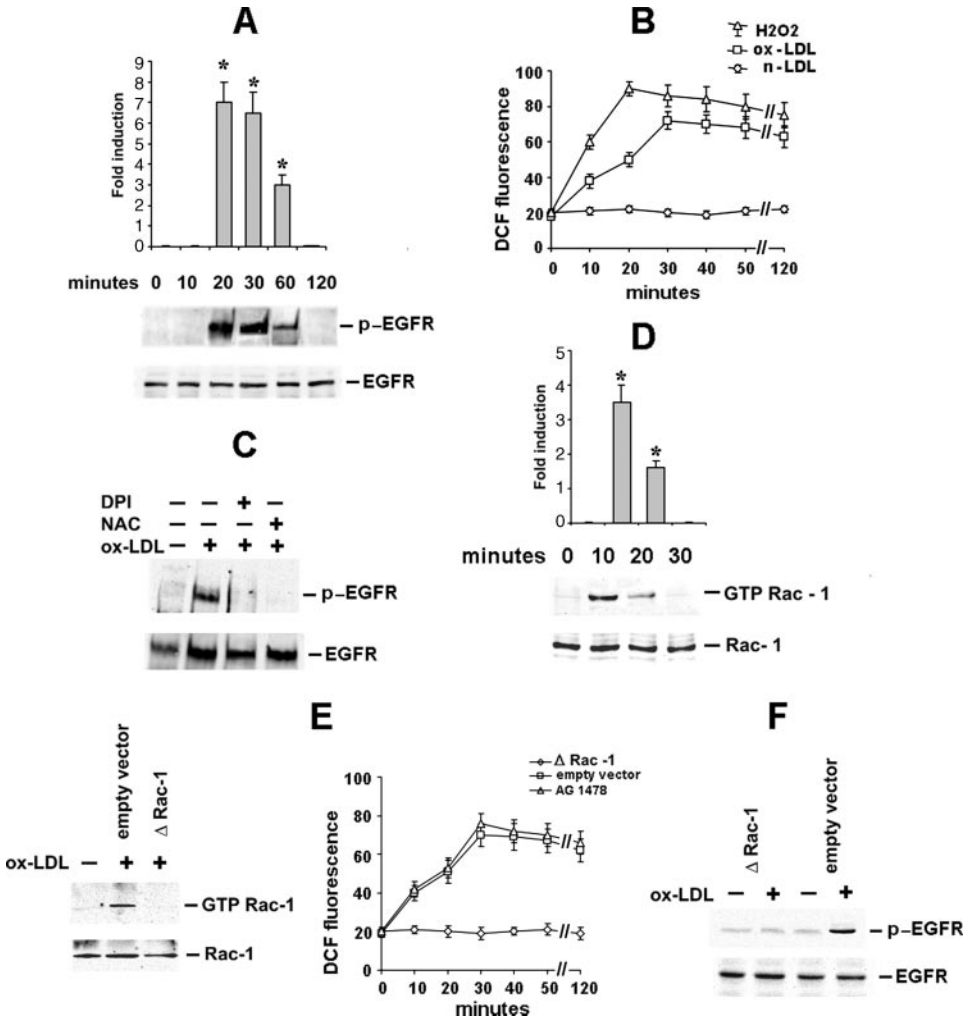

FIGURE 3. **EGFR tyrosine phosphorylation occurs in response to ox-LDL and relies on Rac-1-mediated ROS production.** *A,* time course experiment was performed on ox-LDL-treated MC, and the filter was immunoblotted with an anti-p-EGFR and an anti-EGFR antibody. *B,* kinetics of ox-LDL-induced ROS production. DCF was added to the samples, and fluorescence was measured over a 120-min period. Time 0 represents basal DCF fluorescence. H_2O_2 (100 μM/liter) was used as a positive control. *C,* cell extracts from MC untreated or treated with ox-LDL were subjected to SDS-PAGE. The filter was immunoblotted with an anti-p-EGFR and an anti-EGFR antibody. In selected experiments ox-LDL treatment was combined with NAC (20 mM/liter) or DPI (10 μM/liter). *D,* proteins from ox-LDL-treated samples were pulled down with GST-PAK or directly subjected to SDS-PAGE. The filter was immunoblotted with an anti-Rac-1 antibody. GTP-Rac-1 and total Rac-1 are indicated. *E,* ox-LDL-stimulated MC transfected with an empty vector or with a dominant negative Rac-1 construct (ΔRac-1) were analyzed for Rac-1 activation as above described (*left panel*). MC expressing the empty vector or the ΔRac-1 construct and MC pretreated with AG1478 (250 nM/liter) were analyzed for ROS production (*right panel*). Time 0 represents basal DCF fluorescence. *F,* MC expressing the empty vector or the ΔRac-1 construct were stimulated with ox-LDL and lysed. The filter was immunoblotted with an anti-p-EGFR and with an anti-EGFR antibody. Three different experiments were performed with comparable results.

was calculated by using comparative threshold cycle methods. The β4 primer sequences were as follows: sense (5′-GAA TTC GTT CTA CGC TCT CC-3′) and antisense (5′-GAA TTC TGA GAG ATG TGG GC-3′).

Statistical Analysis—All *in vitro* results are representative of at least three independent experiments performed in triplicate. Densitometric analysis using a Bio-Rad GS 250 molecular imager was used to calculate the differences in the fold induction of protein activation or expression (*, $p < 0.05$, statistically significant between experimental and control values). In selected experiments densitometric analysis was reported in Figs. 1–3 and 5. Significance of differences between experimental and control values were calculated using analysis of variance with Newman-Keuls multicomparison test.

RESULTS

Ox-LDL Induce MC Proliferation—Ox-LDL are implicated in the development of glomerular diseases, including diabetic mesangial proliferation (1). Mesangial cell cycle progression was evaluated in response to ox-LDL. In Table 1 we show that following 48 or 72 h of stimulation with ox-LDL, the proportion of cells in the S phase was increased. Conversely, as reported previously (33), AGE failed to induce cell cycle progression. A dose-response curve (from 10 to 100 μg/ml ox-LDL) indicated that ox-LDL concentrations above 60 μg/ml led to apoptosis. Thus, 50

μg/ml of ox-LDL were used throughout the study. Conversely, n-LDL did not change the percentage of cells in the S phase. Because similar results were obtained after 48 or 72 h of ox-LDL stimulation, 48 h of stimulation was used throughout the study. Cell cycle phases are coordinated by regulatory proteins, including cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors. The expression of cyclin D1 and of the cyclin-dependent kinase inhibitor p21 was therefore assayed in ox-LDL-treated MC. Kinetic analysis (Fig. 1*A*) demonstrated that, consistent with the increase in the proportion of cells in the S phase, the expression of cyclin D1 but not that of p21 was detected. Moreover, to validate the role of the ox-LDL receptor LOX1 in mediating ox-LDL-induced cell cycle machinery, cyclin D1 expression was evaluated in MC pretreated with the blocking LOX1 antibody. The results, reported in Fig. 1*B*, demonstrate that the blockage of LOX1 completely abolished the induction of cyclin D1. Similarly the progression in the cell cycle (Table 2) was prevented by LOX1 blockage.

Akt and Erk1/2 MAPK Signaling Pathways Are Activated in Response to Ox-LDL—MAPK signaling pathway has been reported to be associated with MC proliferation (2). Here, we demonstrate that in response to ox-LDL binding to LOX-1, Erk1/2 MAPK became activated (Fig. 2*A*). Moreover, consistent with data obtained in smooth muscle cells (4), ox-LDL but not n-LDL were also able to trigger Akt activation (Fig. 2*A*). It is known that Akt is involved in survival pathways rather than in mitogenic signals (34). Thus, to evaluate the role of Akt in the survival pathway, MC were transiently transfected with an Akt siRNA sequence (Fig. 2*B*). FACS analysis of MC depleted of Akt demonstrated that these cells failed to progress into the cell cycle (Table 3) and that almost 20% of the cells underwent apoptosis (Table 3), indicating that Akt signaling sustains a survival pathway. Similar results were obtained using a pharmacological inhibitor of Akt (Table 2). To evaluate the contribution of the MAPK signaling pathway in ox-LDL-mediated effects, FACS analysis was also performed in MC pretreated with the MAPK inhibitor PD98059. The results, reported in Table 3, demonstrated that treatment with PD98059 led to a decreased number of cells in the S phase and that, unlike in Akt silenced MC, no apoptotic cells could be detected, indicating that a discrete signaling pathway is activated by MAPK and Akt. As expected, MC treated with PD98059 showed a strong inhibition of Erk1/2 MAPK phosphorylation (Fig. 2*C*).

Ox-LDL-induced EGFR Transphosphorylation Depends on Rac-1-mediated ROS Production—It has been shown that EGFR transactivation contributes to ox-LDL-mediated cell proliferation in different cell types (35). We thus investigated whether EGFR phosphorylation participated with ox-LDL-induced MC hypercellularity. As reported in Fig. 3*A*, kinetic analysis of ox-LDL-treated MC demonstrated a delayed EGFR phosphorylation raising the possibility that EGFR activation may depend on intermediate molecules acting downstream to LOX-1. ROS generation is implicated in the regulation of different cell functions (5). To evaluate the possibility that EGFR activation might depend on ROS production, MC were incubated with dichlorofluorescein. As shown in Fig. 3*B*, ROS generation occurred after 30 min of ox-LDL treatment and was still detectable after 2 h of treatment, whereas n-LDL were ineffective (Fig. 3*B*). To assess the possibility that EGFR phosphorylation relied on ROS production, NAC and DPI were used. Indeed, both treatments completely abrogated the effect ox-LDL-induced EGFR phosphorylation (Fig. 3*C*).

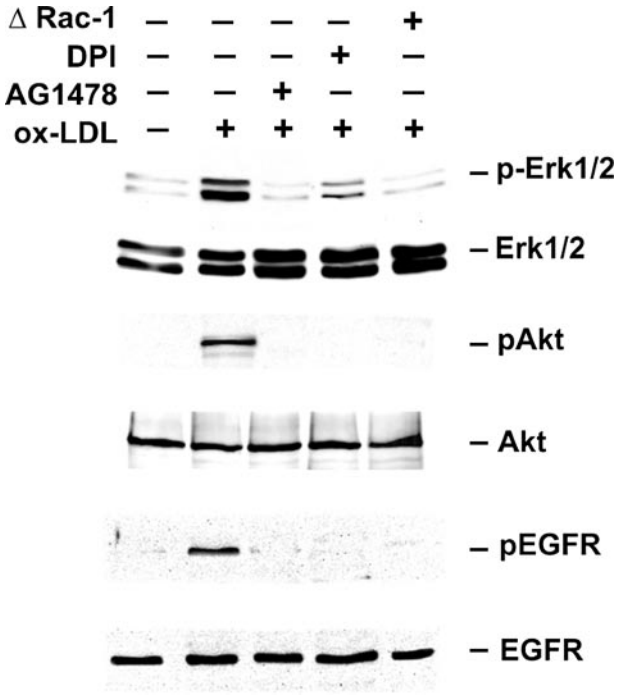

FIGURE 4. **Akt and Erk1/2 MAPK activations depend on Rac-1-mediated ROS production and EGFR tyrosine phosphorylation.** MC expressing the dominant negative Rac-1 construct (ΔRac-1) or MC pretreated with DPI or with the EGFR inhibitor, AG1478, were stimulated or not with ox-LDL and lysed. Cell extracts were subjected to SDS-PAGE, and the filter was immunoblotted with anti-total and anti-p-Erk1/2 MAPK, anti-total and anti-p-Akt, anti-total and anti-p-EGFR antibodies, as indicated. Three different experiments were performed with comparable results.

Because Rac-1 participates in ROS generation (7), the GTP-bound Rac-1 pulldown assay was performed (Fig. 3*D*). The finding that GTP-bound Rac-1 pulldown could be detected in MC challenged with ox-LDL led us to evaluate ROS generation in MC transfected with a dominant negative Rac-1. Data reported in Fig. 3*E* (*right panel*) show that ROS generation was abrogated in dominant negative Rac-1-expressing cells (*left panel*) upon ox-LDL, whereas this effect was retained by MC expressing the vector alone.

To determine the contribution of Rac-1/ROS production in ox-LDL-mediated EGFR phosphorylation, dominant negative Rac-1 MC were used. Indeed, ox-LDL failed to induce EGFR phosphorylation (Fig. 3*F*) in these cells. This finding together with the observation that the AG1478 inhibitor did not affect ROS production (Fig. 3*E*) provides evidence that EGFR activation is downstream to ROS.

Akt and Erk1/2 MAPK Activation Depends on Rac-1-mediated ROS Production and EGFR Phosphorylation—To investigate whether Akt and Erk1/2 MAPK are targets of Rac-1-induced ROS production and EGFR phosphorylation, the activation of both pathways was evaluated in MC pretreated with the AG1478 inhibitor and in the dominant negative Rac-1-expressing cells. The results, depicted in Fig. 4, demonstrate that in both conditions the ox-LDL challenge failed to trigger

TABLE 4

Rac-1-mediated ox-LDL-induced MC cell cycle progression

MC transfected with the empty vector (pEXV) or with the dominant negative Rac-1 construct (ΔRac-1) were serum-starved and stimulated for 48 h with ox-LDL (50 μg/ml) or 10% fetal calf serum (FCS). MC were also pretreated with DPI (10 μM/liter) and processed as above. After stimulation, the cells were fixed with ethanol, and DNA content was evaluated by flow cytometry. The percentage of cells in each phase of the cell cycle was determined by ModFit LT software (Verity Software House Inc.) The numbers are the mean ± S.D. of three independent experiments each performed in duplicate.

Cell cycle phases	Percentage of cells								
	Empty vector (pEXV)			ΔRac-1			DPI		
	Control	Ox-LDL	FCS 10%	Control	Ox-LDL	FCS 10%	Control	Ox-LDL	FCS 10%
G_0/G_1	59 ± 5	49 ± 3	49 ± 3	58 ± 4	60 ± 3	49 ± 3	57 ± 4	58 ± 3	46 ± 3
S	29 ± 4	42 ± 4	41 ± 3	30 ± 3	30 ± 2[a]	40 ± 3	30 ± 3	33 ± 5[a]	46 ± 4
G_2/M	12 ± 2	9 ± 1	10 ± 2	12 ± 1	10 ± 2	11 ± 2	13 ± 1	9 ± 1	8 ± 2

[a] $p < 0.05$, ox-LDL-treated: empty vector expressing cells *versus* ΔRac-1 expressing cells or DPI-treated cells are indicated.

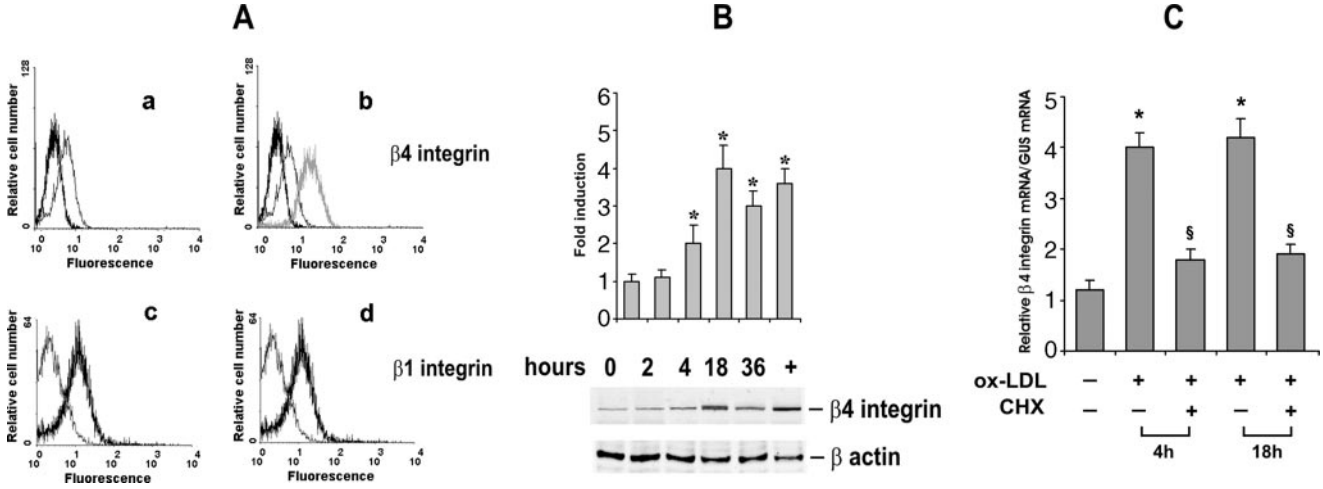

FIGURE 5. **Ox-LDL induce β4 integrin expression in MC.** *A,* MC, untreated (*panels a* and *c*) or ox-LDL-treated for 18 h (*panels b* and *d*) were analyzed for the expression of the β4 (*panels a* and *b*) and β1 (*panels c* and *d*) integrins by FACS analysis (*panels a–d*; *left curve*, preimmune rat or mouse IgG, used as negative controls; *panel b*, *continuous line*, untreated MC; *gray line*, ox-LDL-treated MC). *B,* time course experiment of β4 integrin expression was performed on cell extracts from ox-LDL-treated MC. The normalization of the loaded proteins was performed using an anti-β-actin antibody. Mammary tumor cells (MCF7) were used as positive control (+). *C,* real-time quantitative RT-PCR analysis of β4 integrin mRNA levels (normalized to GUS mRNA) in ox-LDL-stimulated MC in the presence or in the absence of CHX (20 μg/ml). *, $p < 0.05$, ox-LDL-treated MC *versus* control; §, $p < 0.05$, ox-LDL + CHX *versus* ox-LDL. Three different experiments were performed with comparable results.

Akt and Erk1/2 MAPK activation. Similar results were obtained by using DPI (Fig. 4). Both events were associated with the abrogation of EGFR phosphorylation (Fig. 4). Consistent with these results, FACS analysis (Table 4) demonstrated that cell cycle progression was prevented by expressing the dominant negative Rac-1 or by pretreating MC with DPI.

β4 Integrin Expression Is Affected by Ox-LDL—A stringent control on EGFR signaling is exerted by β1 and β4 integrins (13, 16). Diabetes-associated increase of glucose concentrations has been shown to induce, on endothelial cells, the expression of several membrane molecules including β4 integrin (36). Thus, the level of β1 and β4 integrin expression was evaluated on ox-LDL. The results (Fig. 5, *A, panel b,* and *B*) demonstrate that β4 integrin, but not β1 integrin (Fig. 5*A, panel d*), is up-regulated by oxidative stress.

To evaluate the possibility that the increased expression of β4 may rely on a "*de novo*" protein synthesis, real-time PCR (Fig. 5*C*) was performed in MC pretreated with cycloheximide. The observations that ox-LDL increased β4 mRNA expression and that cycloheximide abrogated this effect indicate that β4 integrin is newly synthesized.

β4 Integrin Expression Is Required for Ox-LDL-mediated MC Cell Cycle Progression—To assess the contribution of β4 integrin in ox-LDL-induced MC cell cycle progression, MC were transfected with a β4 integrin mutant, devoid of its cytoplasmic domain (β4L) or with a pSUPERretro vector containing β4-shRNA. The expression of the β4L protein and of the pSUPERretro vector containing β4-shRNA is reported in Fig. 6*A*. As

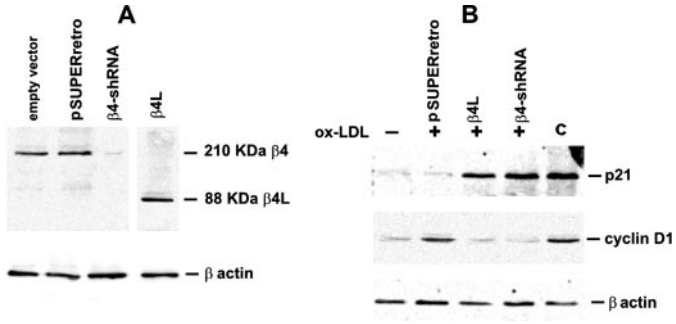

FIGURE 6. **Cyclin D1 and p21 expression in MC expressing the β4L construct or the β4-shRNA.** *A,* MC expressing the β4L construct or the empty vector alone and MC expressing the β4-shRNA or the pSUPERretro vector were lysed and subjected to SDS-PAGE. The filter was immunoblotted with the anti-human β4 integrin antibodies as follows: mAb 450-11 or H-101, recognizing the cytoplasmic and the extracellular domains, respectively. The filter was reprobed with an anti-β-actin antibody. *B,* cell extracts from MC expressing the different constructs, as above described, were analyzed by Western blot with anti-cyclin D1, anti-p21, and anti-β-actin antibodies, as indicated. AGE- or fetal calf serum-treated MC were used as p21- and cyclin D1-positive control (*c*), respectively. Four different experiments were performed with comparable results.

TABLE 5

Effect of ox-LDL on cell cycle progression of MC expressing the β4L or β4-shRNA constructs

MC, transfected with an empty vector (pRC/CMV) or with the β4L mutant and MC expressing β4-shRNA alone or co-expressing both β4-shRNA and full-length β4 integrin, were serum-starved and stimulated for 48 h with ox-LDL (50 mg/ml) or with 10% fetal calf serum (FCS). After stimulation, the cells were fixed with ethanol, and DNA content was evaluated by flow cytometry. The percentage of cells in each phase of the cell cycle was determined by ModFit LT software (Verity Software House Inc.) The numbers are the mean ± S.D. of three independent experiments each performed in duplicate.

Cell cycle phases	Percentage of cells											
	pRC/CMV vector			β4L			β4-shRNA			β4-shRNA + β4 full-length		
	Control	Ox-LDL	FCS 10%	Control	Ox-LDL	FCS 10%	Control	Ox-LDL	FCS 10%	Control	Ox-LDL	FCS 10%
G_0/G_1	52 ± 4	46 ± 3	44 ± 3	55 ± 4	65 ± 3	48 ± 4	58 ± 4	67 ± 3	40 ± 3	59 ± 4	48 ± 3	41 ± 3
S	34 ± 3	44 ± 3	49 ± 4	36 ± 3	27 ± 3[a]	45 ± 3	32 ± 3	25 ± 2[a]	48 ± 3	30 ± 3	35 ± 5[b]	49 ± 4
G_2/M	14 ± 2	10 ± 1	7 ± 2	9 ± 3	8 ± 2	7 ± 2	10 ± 1	8 ± 1	12 ± 2	11 ± 1	7 ± 1	10 ± 2

[a] $p < 0.05$, ox-LDL-treated: pRC/CMV expressing cells *versus* β4L mutant cells or β4-shRNA expressing cells are indicated.
[b] $p < 0.05$, ox-LDL-treated: β4-shRNA expressing cells *versus* β4-shRNA+β4 full-length expressing cells are indicated.

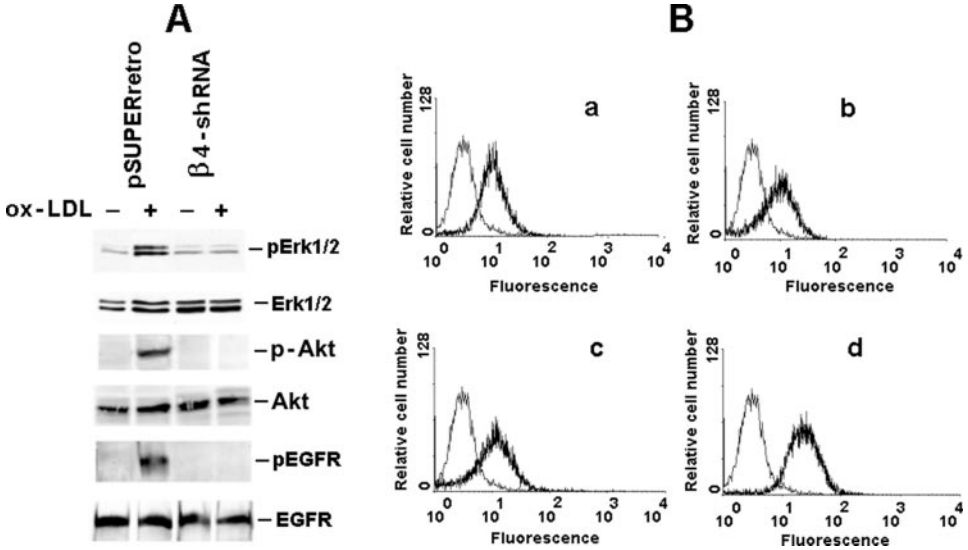

FIGURE 7. **β4 integrin is required for EGFR phosphorylation and for Akt and Erk1/2 MAPK activation upon ox-LDL.** *A,* cell extracts from MC expressing pSUPERretro vector or β4-shRNA, treated or not for 20 min with ox-LDL, were analyzed by Western blot with anti-total and anti-p-Erk1/2 MAPK, anti-total and anti-p-Akt, and anti-total and anti-p-EGFR antibodies, as indicated. *B,* MC stimulated with ox-LDL alone (*panel a*) or in the presence of PD98059 (*panel b*) or AG1478 (*panel d*) and ox-LDL-treated MC endogenously depleted of Akt (*panel c*) were analyzed for β4 integrin expression by FACS analysis (in *panels a–d*, *left curve*: pre-immune rat IgG used as negative control). Four different experiments were performed with comparable results.

shown in Table 5, FACS analysis revealed that both MC expressing the β4L protein and MC endogenously depleted of β4 integrin did not undergo cell cycle progression upon ox-LDL. In addition, as shown in Table 5, transient transfection of the full-length β4 integrin construct in β4-shRNA cells restored cell cycle progression. The results (not reported) of cell cycle analysis, referred to MC transfected with the empty pSUPERretro vector, were comparable with those obtained using the empty vector pRC/CMV.

Cell cycle machinery was also investigated in MC expressing the two different constructs. Both MC expressing the β4L or the β4-shRNA constructs failed to induce cyclin D1 expression, and consistent with the G_0/G_1 cell cycle arrest, ox-LDL treatment led to the induction of p21 (Fig. 6*B*).

β4 Integrin Is Required for Akt and Erk1/2 MAPK Activation and for EGFR Phosphorylation—To gain further insight into the role of β4 integrin in mediating the ox-LDL effects, Akt and Erk1/2 MAPK activation was evaluated in MC endogenously depleted of β4 integrin. As depicted in Fig. 7*A*, we failed to detect both Akt and Erk1/2 MAPK activation, indicating that β4 integrin is essential not only for the induction of cell cycle progression but also for the signaling pathway upstream to the cell cycle machinery. Akt has been referred as a target of β4 integrin in tumor cells (11). The above results together with the observations that endogenous depletion of Akt and Erk1/2 MAPK blockage did not affect ox-LDL-induced β4 integrin expression (Fig. 7*B*, *panels a–c*) identify Akt and Erk1/2 MAPK as targets of β4 integrin-mediated signals in pathological conditions different from cancer.

α6β4 integrin by transactivating EGFR modulates tumor progression (37). To investigate the role of β4 integrin in ox-LDL-mediated EGFR phosphorylation, β4-shRNA MC were examined. The observation that in these cells no EGFR phosphorylation could be detected upon ox-LDL challenge (Fig. 7*A*), and that the re-expression of β4 integrin (data not shown) rescued EGFR phosphorylation, sustains the contribution of β4 integrin in regulating this event. Moreover, the finding that AG1478 did not affect ox-LDL-induced β4 expression further demonstrated that EGFR phosphorylation is downstream to β4 integrin (Fig. 7*B*, *panel d*).

Rac-1-mediated ROS Production Requires β4 Integrin—We further analyzed the effect of endogenous β4 integrin depletion on Rac-1-mediated ROS generation by performing GTP-bound Rac-1 pulldown assay in β4-shRNA MC (Fig. 8*A*). The finding that GTP-bound Rac-1 pulldown was undetectable in the absence of β4 integrin led us to evaluate ROS generation in this condition. Data reported in Fig. 8*B* show that in β4-shRNA MC ROS generation was abrogated, indicating that Rac-1/ROS production strictly depends on the expression of β4 integrin. Consistent with the crucial role of β4 integrin in regulating this event, Rac-1 activation was not affected by β1 integrin knockdown (Fig. 8, *C* and *D*).

β4 Integrin Expression Is Independent on Rac-1-mediated ROS Production—To explore the contribution of Rac-1/ROS production in mediating β4 integrin expression, FACS analysis was performed on dominant negative Rac-1 MC. As shown in

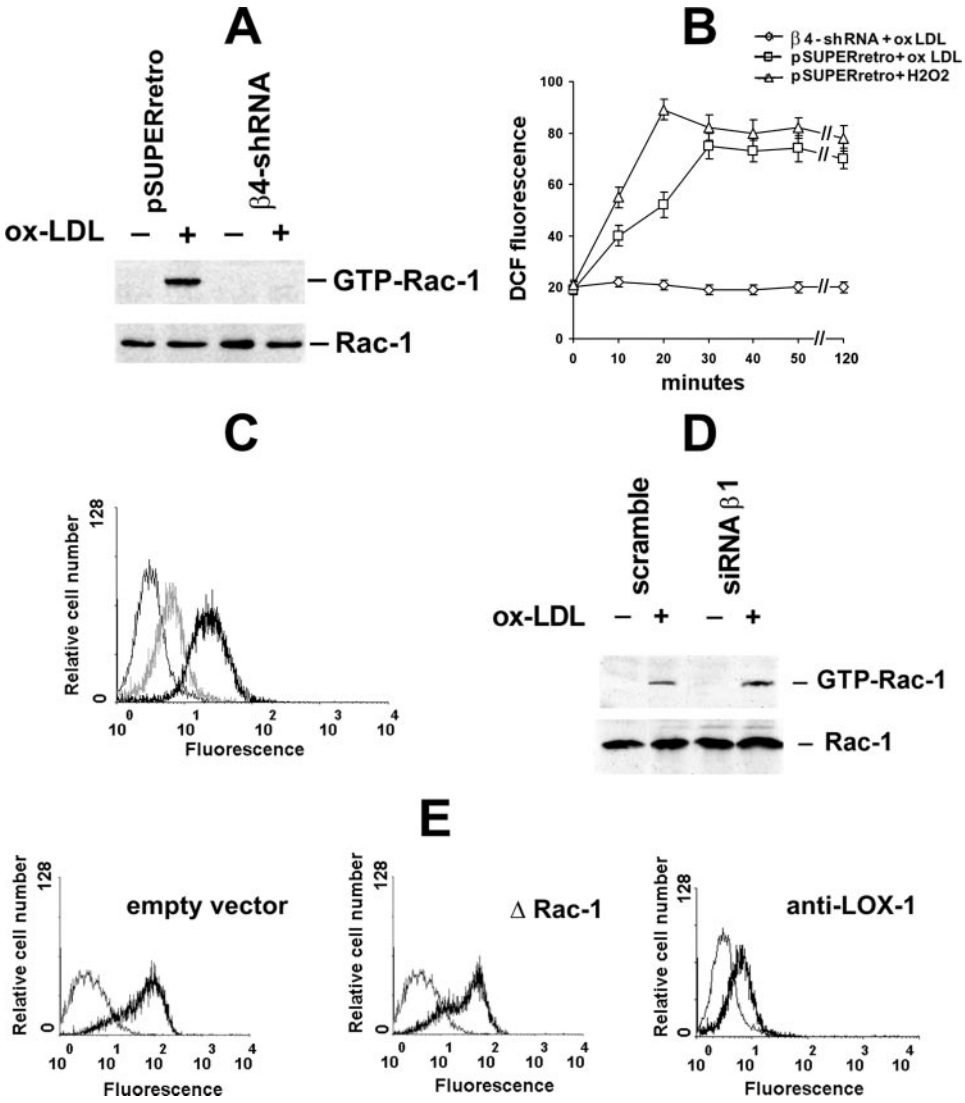

FIGURE 8. **β4 integrin expression is required to Rac-1-mediated ROS production.** *A,* β4-shRNA expressing MC and empty pSUPERretro vector expressing cells challenged with ox-LDL were pulled down with GST-PAK or directly subjected to SDS-PAGE. The filter was immunoblotted with an anti-Rac-1 antibody. GTP-Rac-1 and total Rac-1 are indicated. *B,* MC expressing pSUPERretro vector stimulated with ox-LDL or H_2O_2, and MC expressing β4-shRNA stimulated with ox-LDL were analyzed for ROS generation by DCF fluorescence. Time 0 represents basal DCF fluorescence. *C,* MC transfected with a siRNA β1 (*gray line*) or with a scrambled sequence (*continuous line*) were analyzed by FACS analysis for β1 integrin expression (*left curve,* preimmune rat IgG used as negative control). *D,* MC transfected with a siRNA β1 or with the scrambled sequence (scramble) were challenged with ox-LDL and pulled down with GST-PAK or directly subjected to SDS-PAGE. The filter was immunoblotted with an anti-Rac-1 antibody. GTP-Rac-1 and total Rac-1 are indicated. *E,* MC transfected with the empty vector (pEXV) or with the dominant negative Rac-1 construct or MC pretreated with a blocking anti-LOX-1 antibody were stimulated with ox-LDL and analyzed for the expression of the β4 integrin by FACS analysis (*left curve,* preimmune rat IgG used as negative control). Three different experiments were performed with comparable results.

Fig. 8*E* ox-LDL treatment was still able to induce β4 integrin expression. Similar results were obtained with DPI or NAC treatment (data not shown). Taken together these data indicate that Rac-1 is downstream to β4 integrin and that a Rac-1-independent signaling pathway contributes to ox-LDL-mediated β4 integrin expression. However, that LOX-1 activation regulates β4 integrin expression is indicated by the finding that blockage of LOX-1 prevented the expression of β4 integrin (Fig. 8*E*).

DISCUSSION

The data presented here lead to two major conclusions. (*a*) β4 integrin exerts a stringent control on Rac-1-mediated ROS production, EGFR phosphorylation, and Akt and Erk1/2 MAPK activation leading to cell cycle progression of MC exposed to ox-LDL. (*b*) A Rac-1-independent pathway dictates ox-LDL-mediated β4 integrin expression (see Fig. 9).

Lipid abnormalities in nephrosis have been suggested to play a role in the progression of original glomerular injury to progressive glomerulosclerosis. Indeed, ox-LDL contributes to MC proliferation in the early phase of renal disease (1), and this in turn influences the synthesis and deposition of extracellular matrix proteins leading, eventually, to sclerosis and renal failure. Here we demonstrate that ox-LDL, by activating LOX-1, induce MC cell cycle progression and that this event correlates with the induction of the cell cycle machinery. Our observation is consistent with the expression and activation of specific cyclin and cyclin-dependent kinases detected in various experimental renal diseases, including mesangial proliferative glomerulonephritis (38). The intracellular signaling pathways involved in cell proliferation generally proceed in an orderly fashion by generating early multiple intracellular events propagated into the nucleus for DNA synthesis and cell duplication. Although mitogenic signaling pathways and mechanisms are still poorly defined in renal disease, the activation of the Erk1/2 MAPK pathway has been reported in cortical and isolated glomeruli of rat glomerulonephritis (39). Here we show that ox-LDL induce the activation of the MAPK and the Akt signaling pathways. The crucial role of Akt in protecting MC from ox-LDL-induced cell toxicity is indicated by the observations that silencing Akt led to a dramatic decrease of cell proliferation concomitant with a rise of apoptosis. Moreover, that this effect specifically relies on Akt activation is indicated by the observation that pharmacological inhibition of the Erk1/2 MAPK signaling pathway induces cell cycle arrest without affecting cell survival. Our finding that a neutralizing anti-LOX-1 antibody abrogates the effect of ox-LDL on cell cycle machinery as well as on Erk1/2 MAPK and Akt activation (data no shown) also sustains the role of LOX-1 engagement in mediating these events.

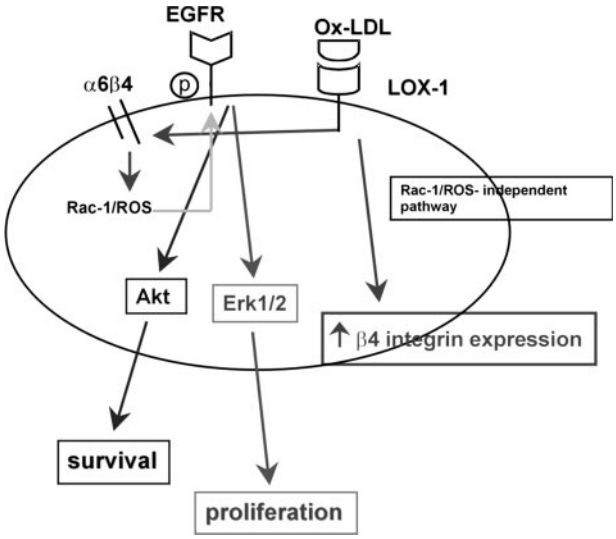

FIGURE 9. **Schematic model of oxidative stress-mediated mesangial cell proliferation and survival.** Oxidative stress elicited in MC exposed to ox-LDL triggers two independent signals as follows: (i) a Rac-1/ROS-dependent pathway, relying on β4 integrin activation and regulating downstream events (EGFR phosphorylation, Erk1/2 MAPK, and Akt activation), that sustains cell cycle progression and cell survival; (ii) a Rac-1/ROS independent pathway leading to increased β4 integrin expression.

There is increasing evidence that oxidative stress or redox-dependent protein modification modulates early events of signal transduction for cell growth and death (5). This study provides evidence that ox-LDL, by ROS production, activate the EGFR/Akt pathway to prevent apoptosis and the EGFR/MAPK pathway to promote cell cycle progression. Various nonspecific factors (40, 41) are able to induce a transient EGFR phosphorylation independently of EGF binding. Moreover, in human endothelial cells, EGFR is known to be a target of ox-LDL (35). Here we show that by ROS production ox-LDL trigger EGFR phosphorylation that controls cell cycle progression. In line with our results, H_2O_2-induced EGFR phosphorylation controls downstream phosphorylation signals and cell proliferation (42). The contribution of redox stress in promoting EGFR tyrosine phosphorylation and its downstream events is also provided by the observation that NAC and DPI prevented both EGFR tyrosine phosphorylation and the signaling cascade leading to MC progression into the cell cycle. The prolonged ligand-induced EGFR phosphorylation seems to mainly depend on H_2O_2 production (43). Our results show that, unlike inhibition of intracellular redox state, by means of NAC or DPI, specific inhibition of EGFR phosphorylation did not prevent ox-LDL-induced ROS production suggesting the involvement of an EGF-independent mechanism. Consistently, the blockage of EGF by a specific antibody did not prevent ox-LDL-mediated effects (data not shown).

It is a recent notion that the family of Ras-related small GTP-binding proteins may function as general regulators of the intracellular redox states (6). More importantly, Rac-1 mediates the transient rise of ROS after cell stimulation in different cell types (7). Here we first report that Rac-1 takes a specific role in the production of ROS after exposure of MC to ox-LDL. Several studies have implicated Rac-1-mediated ROS production in a variety of cellular responses, in particular in endothelial cells (7). An important issue raised by our experiments deals with the contribution of Rac-1-mediated ROS production in EGFR activation as well as in the signaling cascade promoting MC cell cycle progression upon ox-LDL. Susceptibility to diabetic nephropathy mainly relies on oxidative stress (44), and increased levels of ROS in association with up-regulation of the $gp91^{phox}$ and Rac-1 expression have been reported in diabetes (45). Thus, our data add further insight into the molecular mechanisms promoting early MC hypertrophy in diabetes-associated renal disease.

Mitogenic signals emanated by RPTK involve, as a critical downstream event, ROS generation. Integrin signaling is essential for cell growth and differentiation and is constantly integrated with growth factor signaling. Indeed, ROS are generated after integrin engagement to regulate cell adhesion (46). Here we first show that β4 integrin is required for ox-LDL-mediated ROS production, EGFR phosphorylation, and for downstream events regulating MC cell cycle progression. The small GTPase Rac-1 is a central component of signaling machinery downstream of adhesion molecules (47). Our finding that Rac-1 activation requires the expression of β4 integrin reinforces the idea that Rac-1 lies downstream of adhesion molecules also in the redox cascade triggered by ox-LDL. Some of the survival signals, such as those proceeding through PI3-K/Akt, are activated jointly by integrin and RPTKs, explaining why optimal cell survival often requires ligation of both types of receptors. Here, we also report that in MC challenged with ox-LDL joint β4 integrin/EGFR signaling controls both cell survival and cell cycle progression.

The integrin system is characterized by a high degree of complexity and specificity. Because the expression of integrin is mainly regulated during development and differentiation, each cell type has its own distinctive repertoire of integrins. However, hyperglycemia has been shown to induce β4 integrin expression in endothelial cells (36). Our data demonstrated that oxidative stress can modulate the expression of β4 integrin; however, the kinetics of β4 integrin expression clearly ruled out the possibility that ox-LDL-induced Rac-1 activation relies on this event.

Although further studies are required to define the mechanistic events contributing to β4 integrin expression, the finding that CHX treatment affects β4 mRNA expression indicates that, in our experimental conditions, β4 integrin depends on *de novo* protein synthesis. However, it can be substantially ruled out by kinetic experiments.

Both injury and EGF enhance the expression of α6β4 mRNA, whereas migration enhances the β4 synthesis (48). In addition Song *et al.* (49) showed that depletion of EGFR prevents β4 integrin mRNA expression. Our findings that EGF blockage did not prevent ox-LDL-mediated effects and that, after inhibition of EGFR activation, ox-LDL still retain the ability to induce the expression of β4 integrin indicate that different mechanisms may account for our results.

Hyperlipidemia is noted in various experimental renal diseases, including model of diabetes, adriamycin-induced nephrotic syndrome, and Dahl salt-sensitive hypertensive rats (50). The results of this study identify a novel molecular mechanism by which oxidized lipids can induce mesangial hypertro-

phy in the early stage of renal disease. In particular we provide evidence that a stringent control is exerted by β4 integrin on Rac-1/ROS-mediated signaling events leading to progression of MC into the cell cycle, a notion that opens new avenues for pharmacological intervention in oxidative stress-induced renal disease.

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Oxidative Stress-mediated Mesangial Cell Proliferation Requires RAC-1/Reactive Oxygen Species Production and β4 Integrin Expression

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