Low density lipoprotein receptor-related protein (LRP)\(^2\) is a multifunctional endocytic receptor implicated in the modulation of a number of cellular processes, including the turnover of proteases and the degradation of extracellular matrix proteins. As such, it can play a key role in the control of fibrosis. The aim of this investigation was to ascertain whether the anti-fibrotic effects exerted by the angiotensin-converting enzyme inhibitor (ACE-I) perindoprilat on macrophage-conditioned medium (MPCM)-injured human mesangial cells can be modulated by this receptor. Addition of receptor-associated protein to MPCM-injured mesangial cells with and without ACE-I increased the amount of tissue plasminogen activator protein detected in mesangial cell culture supernatants without affecting the protein levels of plasminogen activator inhibitor-1. The ability of ACE-I to reduce fibronectin was diminished in the presence of receptor-associated protein. ACE-I induced an increase in mesangial cell MMP9 mRNA, but reduced the MMP9 enzyme activity detected in mesangial cell supernatants. Mesangial cell lysates from ACE-I-treated cells were able to bind immobilized fibronectin at higher dilutions than cell lysates from untreated cells. Flow cytometry showed that MPCM induced an increase in LRP surface expression in mesangial cells over that in control cells and that this expression was further increased by ACE-I treatment. The increase in LRP expression in response to ACE-I was also observed by Western blotting. Northern blot analysis of RNA extracted from cells following a 24-h exposure to MPCM with and without ACE-I demonstrated that there was no change in LRP mRNA expression upon ACE-I treatment. In conclusion, we show that ACE-I treatment is able to modulate mesangial cell-surface expression of LRP, providing an additional mechanism whereby ACE-Is can mediate anti-fibrotic actions independent of their hemodynamic actions.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human mesangial cells were cultured from glomerular explants processed from the normal poles of nephrectomized human kidneys with renal carcinoma using standard serial sieving techniques (4). All donors had given consent for post-surgical use of their kidneys, and procedures on donated kidneys had been approved by an ethical committee. The cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 20% heat-inactivated fetal calf serum (Sigma), 5 \(\mu\)g/ml bovine insulin (Sigma), 100 units/ml penicillin (Invitrogen), 100 \(\mu\)g/ml streptomycin (Invitrogen), and 2 mM glutamine (Invitrogen). Mesangial cells from passages 2–10 were cultured in Costar Corning 75-cm\(^2\) flasks. For experiments, mesangial cells were grown to confluence in Costar...
Corning 24-well plates or 25-cm² flasks and then rendered quiescent in RPMI 1640 medium containing 0.5% fetal calf serum for 48 h prior to use. All experiments were carried out in RPMI 1640 medium and 0.5% fetal calf serum. Cells of the human monocyte/macrophage cell line U937 (ECACC 85011440) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine.

Preparation of U937 Cell-conditioned Medium—U937 cell-conditioned medium was prepared as described previously (4).

Preparation of Cell Lysates—After removal of tissue culture supernatants, cell monolayers were washed with phosphate-buffered saline, scraped into 250 μl of 1% Igepal CA-630 (Sigma) in wash buffer (phosphate-buffered saline containing 0.3 mM NaCl and 1% Tween 20), and then incubated at room temperature for ~30 min. The cell scrapings were transferred to 2-ml tubes, sonicated with a 5-s burst, and centrifuged for 1 min at 11,600 × g. Sonication and centrifugation were repeated, and the lysate supernatants were assayed for total cell protein.

Culture of Mesangial Cells in the Presence of MPCM—Confluent, quiescent mesangial cells were exposed to a 50% solution of MPCM in the presence or absence the ACE-I perindoprilat (40 μM; a gift from Servier, Neuilly-sur-Seine, France) or in the presence or absence of 500 nM receptor-associated protein (RAP; Calbiochem). The cultures were maintained under the various conditions for 1 or 3 days. The tissue culture supernatants were harvested and stored at ~20 °C for subsequent analysis. For Northern analysis, mesangial cells were exposed to 50% MPCM with or without additions for ~18 h prior to RNA processing.

Fibronectin Enzyme-linked Immunosorbtent Assay (ELISA)—Culture supernatants were assayed for fibronectin as described previously (4).

LRP Binding Assay—This assay was adapted from the protocol described by Salicioni et al. (3). 100 μl of 10 μg/ml human plasma fibronectin (Calbiochem) was immobilized on Costar Corning 96-well microtiter plates overnight at 4 °C. 10 μg/ml bovine serum albumin (BSA; Sigma) was coated on parallel plates to serve as a negative control. Nonspecific binding sites were blocked with 2% (w/v) BSA for 1 h. Cell lysates from mesangial cells exposed to MPCM with or without ACE-I were added to the wells at dilutions of 1:10, 1:50, and 1:100 and incubated overnight at 37 °C in pH 7.6 developer buffer (50 mM Tris-buffered saline containing 0.5%Tween 20 for 1 h. The membranes were then washed once with Tris-buffered saline containing 0.5% Tween 20 for 1 h. The membranes were then washed once with Tris-buffered saline containing 0.5% Tween 20 prior to incubation with an anti-LRP antibody (1:100 dilution) for at least 2 h at room temperature. After three more washes, the membranes were incubated with alkaline phosphatase-labeled rabbit antimouse immunoglobulins for at least 2 h at room temperature. The membranes were washed an additional three times before addition of chromogenic substrate (SIGMAFAST® 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets).

Protein Determination—The protein content of cell lysates dissolved in 1% Igepal was determined with a commercial Bio-Rad DC protein assay using BSA standards according to the manufacturer’s instructions.

Gelatin Zymography—Culture supernatants were mixed 1:1 with nondenaturing sample buffer and resolved at 4 °C on 8% SDS-polyacrylamide gels containing 2.5 mg/ml gelatin (electrophoresis grade, 300 bloom; Sigma). The gels were washed with 2.5% Triton X-100 (Sigma) for ~1 h, after which they were incubated overnight at 37 °C in pH 7.6 developer buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM CaCl₂, and 0.05% (w/v) Brij). The gels were then stained with Brilliant Blue R® (Sigma) and destained with a solution of 40% methanol and 10% acetic acid. Destained gels were dried, and bands of lysis were scanned using a Bio-Rad Model GS-700 imaging densitometer.

Fibrinogen Zymography—Culture supernatants were mixed 1:1 with nondenaturing sample buffer and resolved at 4 °C on 11% SDS-polyacrylamide gels containing 12 mg of fibrinogen (Calbiochem), 10 units of plasminogen (Calbiochem), and 10 units of thrombin (Calbiochem) per 10 ml of gel. Following electrophoresis, the gels were washed, incubated in developer buffer, and stained as described above for gelatin zymography.

Northern Blotting—Northern analysis was carried out using a previously described method (4).

Reverse Transcription (RT)-PCR—0.5-μg aliquots of total RNA were reverse-transcribed using an avian myeloblastosis virus reverse transcription system (Promega, Southampton, UK) according to the manufacturer’s instructions. The resulting cDNA was amplified using ReddyMix™ PCR Mastermix (ABgene, Surrey, UK) and 50 pmol of specific sense and antisense primers. Thermocycling conditions were optimized for

Flow Cytometry—Confluent, quiescent mesangial cells were stimulated with MPCM with and without ACE-I for 18 h. Cell monolayers were washed with PBS buffer (phosphate-buffered saline containing 0.1% BSA and 0.05% sodium azide), after which the cells were detached from the flask surface using trypsin/EDTA (Invitrogen). The cells were then washed with 5 ml of PBS buffer before being resuspended in either 50 μl of anti-CD91 antibody (1:10 dilution) or phosphate-buffered saline only. After a 40-min incubation on ice, the cells were washed twice with PBS buffer and then resuspended in 50 μl of fluorescein isothiocyanate-labeled goat F(ab′)₂ anti-mouse Ig antibody (1:50 dilution; DakoCytomation). Fluorescein isothiocyanate-labeled secondary antibody alone was used as a negative control. After a 30-min incubation on ice, the cells were washed twice with PBS buffer and resuspended in 500 μl of PBS buffer for analysis of median fluorescence on a FACScan flow cytometer (BD Biosciences).

Western Blotting—Cell monolayers from each well were scraped into 150 μl of nonreducing sample buffer. The samples were boiled for 5 min and resolved on 7% SDS-polyacrylamide gels. The gels were blotted onto nitrocellulose membranes and immunostained. Briefly, the membranes were blocked with a 2% solution of BSA in Tris-buffered saline containing 0.5% Tween 20 for 1 h. The membranes were then washed once with Tris-buffered saline containing 0.5% Tween 20 prior to incubation with an anti-LRP antibody (1:100 dilution) for at least 2 h at room temperature. After three more washes, the membranes were incubated with alkaline phosphatase-labeled rabbit antimouse immunoglobulins for at least 2 h at room temperature. The membranes were washed an additional three times before addition of chromogenic substrate (SIGMAFAST® 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets).

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Perindoprilat Modulates LRP Expression

RAP is the most avid ligand of LRP (9) and as such binds to LRP at the expense of other ligands. To examine whether ACE-Is play a role in LRP-mediated binding or endocytosis of tPA, RAP (500 nM) was added to MPCM-injured cells in the presence or absence of the ACE-I perindoprilat. As observed previously (4), the culture supernatants from MPCM-injured cells treated with ACE-I contained lower tPA levels than those from untreated cells. Addition of RAP resulted in the accumulation of tPA in mesangial cell supernatants, approximately doubling the levels compared with non-RAP-treated cells (Fig. 1A). Supernatant tPA levels were typically 8.37 ± 0.82 ng/ml (0.1 ng/μg of cell protein) in MPCM-injured cells and 1.63 ± 0.17 ng/ml (0.012 ng/μg of cell protein) in control cells, whereas cell lysate tPA levels were typically 27.1 ± 4.4 ng/ml (0.32 ng/μg of cell protein) in injured cells and 6.39 ± 1.1 ng/ml (0.07 ng/μg of cell protein) in control cells. RAP did not have the same cumulative effects on plasminogen activator inhibitor-1 levels. However, RAP did reverse the perindoprilat-induced plasminogen activator inhibitor-1 lowering effects that we reported previously (4): MPCM, 2.81 ± 0.05 ng/μg of protein; MPCM + perindoprilat, 2.44 ± 0.05; MPCM + RAP, 2.81 ± 0.04; MPCM + perindoprilat + RAP, 2.9 ± 0.2; and medium alone, 2.4 ± 0.18 (n = 2).

The tPA assay data were supported by fibrinogen gel zymography on culture supernatants, which showed an accumulation of tPA activity in the presence of RAP (Fig. 2). Mesangial cell lysates were analyzed for the presence of immunoreactive tPA to assess whether the apparent ACE-I-induced reduction of tPA levels in culture supernatants could be accounted for by binding to, or uptake by, mesangial cells. The results demon-

ELISAs—Imulyse™tPA ELISA and TintElize™plasminogen activator inhibitor-1 ELISA (Alpha Laboratories Ltd., Hampshire, UK) were carried out according to the manufacturer’s instructions.

Statistics—Mesangial cell fibronectin levels were corrected for cell protein. Fibronectin levels are expressed as a percentage of the levels obtained with MPCM. Representative autoradiographs of Northern blots, photographs of agarose gels, or zymograms are shown, but densitometric analysis incorporated data from all experiments. Results are expressed as means ± S.E. For comparison of means between two groups, an unpaired t test was employed. To compare values between multiple groups, an analysis of variance with Bonferroni’s correction was applied. Statistical significance is defined as p < 0.05.

RESULTS

FIGURE 1. Effect of RAP on ACE-I-treated mesangial cell tPA levels. Mesangial cells were treated in the presence or absence of ACE-I and/or RAP. Culture supernatants (A) and cell lysates (B) were analyzed for tPA by ELISA. Results show means ± S.E. *, p = 0.002 versus MPCM; †, p < 0.02 versus MPCM and MPCM + ACE-I (n = 4); **, p = 0.03 versus MPCM; †, p < 0.04 versus MPCM (n = 4; not significant between MPCM and MPCM + RAP or MPCM + ACE-I + RAP). Med, medium alone.

FIGURE 2. Effect of RAP and ACE-I treatment on tPA activity. Culture supernatants from mesangial cells treated in the presence or absence of ACE-I and/or RAP were analyzed for active tPA by fibrinogen zymography. A zymogram representative of four experiments is shown. Med, medium alone.
strated that tPA levels in the lysates from cells treated with ACE-I were higher than those from untreated cells (Fig. 1B), indicating that uptake of tPA by mesangial cells was enhanced in the presence of ACE-I. As observed in the supernatants, lysate tPA levels were also higher in RAP-treated cells than in cells treated with MPCM alone. However, there was no significant difference in lysate tPA levels in ACE-I-treated cells regardless of whether they had been treated with RAP or not.

The ability of perindoprilat to decrease MPCM-injured mesangial cell fibronectin levels was diminished in the presence of RAP, although RAP itself appeared to have no effect on the fibronectin levels in non-ACE-I-treated cells (Fig. 3). The fact that fibronectin levels were not further reduced in the presence of accumulated tPA suggests that tPA itself is not directly responsible for the observed fibronectin degradation as a result of its own protease activity or indirectly via plasmin and the subsequent activation of zymogens such as MMP2. Rather, it suggests that uptake of tPA results in the degradation of fibronectin perhaps via a mechanism downstream of tPA binding. It is possible that RAP-mediated inhibition of tPA binding results in reduced receptor signaling and diminution of the consequent downstream events that ultimately result in fibronectin degradation. We have observed previously that addition of exogenous tPA (100 ng/ml = 40 units/ml = 1.5 nM) to MPCM-stimulated mesangial cells resulted in a 15.34 ± 2.1% reduction in fibronectin levels.3 In this study, the levels of accumulated tPA were of the order of 20 ng/ml and therefore probably insufficient to induce observable fibronectin degradation.

TPA is known to signal as a cytokine via LRP, causing increased transcription of MMP9 (10, 11). RT-PCR was carried out to assess mesangial cell expression of MMP9 and MMP2 mRNAs in response to ACE-I treatment. RT-PCR demonstrated that MMP9 mRNA levels were up-regulated in response to ACE-I (MPCM, 0.662 ± 0.105 arbitrary densitometric units; MPCM + ACE-I, 1.35 ± 0.27; and medium alone, 0.61 ± 0.2; *p < 0.03 versus MPCM (n = 4)), whereas MMP2 mRNA levels appeared unaffected (Fig. 4A). However, gelatin zymography on culture supernatants from MPCM-injured, ACE-I-treated mesangial cells unexpectedly showed lower MMP9 activity compared with supernatants from non-ACE-I-treated cells (with reduction in the active form of MMP9 being particularly pronounced) (Fig. 4B). Co-incubation of MPCM-injured mesangial cells with and without ACE-I in the presence of RAP was able to at least restore ACE-I-reduced MMP9 activity (Fig. 4B). Because LRP can also function as a scavenger for MMP9, clear evidence of increased LRP expression was observed following treatment (Fig. 5A), whereas MMP9 levels did not increase following treatment (Fig. 5B). This increase in MMP9 was abolished by the addition of RAP (Fig. 5B). The ability of perindoprilat to decrease MPCM-injured mesangial cell fibronectin levels was diminished in the presence of RAP, although RAP itself appeared to have no effect on the fibronectin levels in non-ACE-I-treated cells (Fig. 3). The fact that fibronectin levels were not further reduced in the presence of accumulated tPA suggests that tPA itself is not directly responsible for the observed fibronectin degradation as a result of its own protease activity or indirectly via plasmin and the subsequent activation of zymogens such as MMP2. Rather, it suggests that uptake of tPA results in the degradation of fibronectin perhaps via a mechanism downstream of tPA binding. It is possible that RAP-mediated inhibition of tPA binding results in reduced receptor signaling and diminution of the consequent downstream events that ultimately result in fibronectin degradation. We have observed previously that addition of exogenous tPA (100 ng/ml = 40 units/ml = 1.5 nM) to MPCM-stimulated mesangial cells resulted in a 15.34 ± 2.1% reduction in fibronectin levels.3 In this study, the levels of accumulated tPA were of the order of 20 ng/ml and therefore probably insufficient to induce observable fibronectin degradation.

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To confirm the role played by LRP in the anti-fibrotic actions of ACE-I, we wished to assess whether ACE-I treatment affects the surface expression of LRP. LRP is known to have a fibronectin-binding domain within its structure (3). This property of LRP was exploited to see whether increased levels of LRP could be detected in mesangial cell lysates following ACE-I treatment. Our data demonstrated that lysates from ACE-I-treated cells were able to bind to immobilized fibronectin at higher dilutions than lysates from non-ACE-I-treated cells (Fig. 5A). (At the 1:50 dilution, lysates from ACE-I-treated cells bound signifi-
significantly more fibronectin ($p < 0.03$) compared with non-ACE-I-treated cells. However, at the 1:100 dilution, there was no significant difference in the binding between treatments ($\pm$ ACE-I) in the presence of $\alpha_2$-macroglobulin.

Flow cytometry using antibodies against LRP demonstrated that MPCM-injured cells bound 37% more anti-LRP antibody than did control cells but that cells treated with ACE-I bound 50% more than did control cells (Fig. 6A). Western blotting also reflected this small increase in LRP protein expression in response to ACE-I treatment (Fig. 6B). Northern blotting carried out on RNA extracted from cells following a 24-h exposure to MPCM in the presence or absence of ACE-I demonstrated that there was no change in LRP mRNA message expression at this time point (Fig. 7), possibly suggesting that ACE-I exerted its effect at the level of protein expression.

DISCUSSION

Until very recently, tPA had been regarded as an important component of the plasminogen activator system...
involved in the attenuation of extracellular matrix accumulation, leading to the reduction of fibrosis after renal injury. Indeed, it was in such a capacity that we tentatively ascribed the role of tPA when we demonstrated that the anti-fibrotic effects of ACE-I in mesangial cells are modulated via the bradykinin/plasminogen activator system axis (4). A number of studies have shown that the role of tPA is far more complex, and as a result, the functional role of tPA has been dramatically revised. It is now believed that tPA also plays an important, plasmin-independent, stimulatory, cytokine-like role responsible for a diverse number of physiological functions, including the regulation of endothelial cell proliferation (13), modulation of neuron apoptosis (14), and transcriptional up-regulation of MMP9 (10, 11). It must be said, however, that the multifarious plasmin-dependent and independent functions of tPA are not mutually exclusive.

Among its many functions, LRP is able to control the “fibrotic status” of its immediate environment. It is involved in the catabolism of extracellular matrix protein such as fibronectin (3) as well as the homeostasis of proteases such as tPA and MMP9 (1). LRP-deficient murine embryonic fibroblasts have been shown to exhibit increased levels of cell-surface fibronectin without the biosynthesis of the matrix protein having been altered (3). Similarly antagonism of LRP with RAP has been shown to increase fibronectin accumulation in wild-type fibroblasts. Transfection of full-length LRP into deficient cells has also been shown to decrease fibronectin levels (3). In this study, we have demonstrated that treatment with ACE-I resulted in altered LRP expression, which may have occurred post-transcriptionally because no changes in LRP mRNA levels were observed 24 h post-treatment. The apparent increase in surface expression of LRP as seen in the presence of MPCM compared with the control (medium alone) may be attributed to a decreased rate of receptor cycling that can occur in the presence of an injurious agent (15), in this case, MPCM. This phenomenon may also contribute to the observed accumulation of fibronectin in the presence of MPCM, as reduced cycling would ultimately delay fibronectin degradation. ACE-I treatment may therefore facilitate an increase in LRP cycling rates or alter the subcellular distribution such that receptors are more rapidly translocated from the endoplasmic reticulum to the surface of the cell, such as has been found previously in vascular smooth muscle cells following treatment with epidermal growth factor and platelet-derived growth factor (15).

Studies have shown that treatment of murine fibroblasts with RAP causes an accumulation of MMP9 due to inhibition of the ability of LRP to scavenge MMP9 (11). In this study, we did not observe an accumulation of MMP9 in the presence of RAP, but rather an accumulation of tPA, suggesting that in our system RAP was blocking the binding and endocytosis of tPA and thereby the subsequent signaling required to up-regulate the expression of MMP9. We speculate that these increased levels of intracellular tPA could be the consequence of a RAP-induced deceleration of the receptor cycling process, resulting not only in an accumulation of extracellular tPA, but also in increased intracellular tPA as a result of a reduced rate of tPA degradation.

We observed a paradoxical decrease in MMP9 activity in ACE-I-treated, MPCM-injured mesangial cells, which was at least restored upon treatment with RAP. This suggests that increased MMP9 mRNA expression, as well as resulting in increased MMP9 mRNA expression, may also be responsible for an increased rate of MMP9 protein scavenging from the culture supernatants. This observation suggests that a dynamic balance exists between tPA binding and signaling, leading to the up-regulation of MMP9 expression, and tPA and MMP9 endocytosis, leading to clearance of the proteases via the same receptor. MMP2 mRNA levels did not appear to be affected by ACE-I treatment. The change in enzyme activity therefore probably represents a post-translational change in preformedzymogen levels. Whether the ACE-I-mediated effects on MMP9 expression are the result of a direct action of ACE-I molecules on LRP or are the consequent downstream effects of ACE-I-induced tPA acting as a cytokine via LRP remains to be determined.

The dose of 40 μM perindoprilat used in this study was based upon a dose-response curve carried out for a previous study (4). This equates to a concentration of 14.7 μg/ml, which is higher than the peak plasma concentration (C_{max}) of either perindopril (prodrug) or perindoprilat typically observed following a single 4-mg dose in a human subject (64 ng/ml and 4.7 mg/ml, respectively) (16), although patients can receive up to 8 mg/day. However, direct in vivo and in vitro comparisons are problematic because the pharmacokinetics of a drug in cell culture will be different from those of the same drug in a human subject. Moreover, patients are dosed daily over long periods of time, whereas the cells in this study were exposed to a single dose for 1 or 3 days. Whether the observed in vitro effects also occur in human patients receiving perindopril is still to be fully determined. However, this study has identified LRP as a possible target of ACE-I action and provides a platform for future research.

In conclusion, we have demonstrated for the first time that ACE-I treatment results in the modulation of mesangial cell-surface expression of LRP, potentially allowing for increased fibronectin catabolism and tPA binding and signaling, beneficial anti-fibrotic effects that are independent of ACE-I hemodynamic actions.

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