The role of bradykinin in the antifibrotic actions of perindoprilat on human mesangial cells

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Background. Angiotensin-converting enzyme inhibitors (ACE-I) protect against the development of glomerulosclerosis using mechanisms partly dissociated from their systemic antihypertensive action. The aim of the current study was to delineate the mechanism of action underlying the antifibrotic effects of the ACE-I perindoprilat in the context of macrophage-mediated scarring in human mesangial cells.

Methods. Mesangial cells were treated with macrophage-conditioned medium (MPCM) in the presence or absence of the ACE-I perindoprilat.

Results. Forty µmol/L perindoprilat reduced MPCM-induced mesangial cell fibronectin levels by 19.4 ± 0.6% (P < 0.001). Immunoprecipitation of 35S-methionine biosynthetically labeled fibronectin and Northern analysis suggested that the decrease in fibronectin levels was not caused by reduced synthesis. MPCM stimulated the production of matrix metalloproteinases (MMP) 2, 3, and 9 in mesangial cells; however, these were not significantly altered by ACE-I treatment, and neither was production of their tissue inhibitor of metalloproteinases (TIMP-1). Addition of exogenous bradykinin to MPCM-treated mesangial cells resulted in a 22.5 ± 1.4% (P < 0.02) reduction in secreted fibronectin levels, while semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) and Southern blotting demonstrated that bradykinin B2 receptor expression was upregulated by 71 ± 30% in MPCM-stimulated mesangial cells in response to ACE-I treatment (P = 0.032). Moreover, the bradykinin B2 receptor antagonist HOE 140 attenuated the beneficial effects of perindoprilat. MPCM-stimulated mesangial cell protein expression levels of plasminogen activator system components tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1) were altered after treatment with ACE-I.

Conclusion. These results suggest that ACE-I-induced renoprotection, in the context of macrophage-stimulated mesangial cell scarring, is mediated, at least in part, via the actions of bradykinin.

Key words: ACE inhibitor, fibronectin, bradykinin, bradykinin B2-receptor, tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA), plasminogen activator inhibitor-1 (PAI-1).

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Angiotensin-converting enzyme inhibitors (ACE-I) have proved to be effective in reducing proteinuria and deposition of extracellular matrix within the kidney, thereby attenuating the progression of renal failure in patients with a variety of renal diseases [1–6]. These renoprotective effects are greater than those seen with other antihypertensive agents, including calcium channel blockers [7]. This has led to the speculation that the renoprotective actions of ACE inhibitors are additional to any beneficial effects arising from their systemic antihypertensive actions [6, 8, 9]. Experimentally, ACE inhibitors have been shown to lower the intraglomerular capillary hypertension, which is thought by many to be etiologically important in the development of glomerulosclerosis and chronic renal failure. Such effects on intraglomerular hemodynamics may explain the additional renoprotective effects seen with this class of antihypertensive agents. However, nonhemodynamic renoprotective actions are also possible. These may be explained by the intrarenal actions of the renin-angiotensin system (RAS). In vivo transfection of genes for renin and angiotensinogen into glomerular cells has been shown to induce extracellular matrix expansion in glomeruli, with concomitant expression of mesangial cell smooth muscle actin [10]. Angiotensin II, the product of ACE activity, has been shown to stimulate matrix protein synthesis via the transforming growth factor β (TGFβ) axis [11], and to promote mesangial cell hypertrophy and hyperplasia [11]. Modulation of the local RAS, and particularly, reducing Ang II generation via ACE inhibition, might therefore be expected to reduce the tendency for glomerular extracellular matrix production, and hence, the development of glomerulosclerosis.

Because ACE also catalyzes the degradation of bradykinin [12], it is thought that ACE-I may, in addition, exert their beneficial actions by protecting endogenously produced kinins from degradation. The resulting enhanced local levels of peptide may then contribute to the observed beneficial effects [13].

While in vivo studies can focus on the final outcome of the beneficial effects of ACE inhibitor treatment, the mechanisms underlying the protective effects are difficult...
to define because contributions from the modulation of renal hemodynamics can never be discounted. We have previously demonstrated that macrophages and their secreted products are able to up-regulate mesangial cell matrix production in vitro. Such observations provide potential mechanisms whereby an influx of macrophages into the glomerulus in vivo may promote glomerulosclerosis [14].

The aim of the current study was to delineate the mechanism of action underlying the renoprotective, or more specifically, antifibrotic effects of ACE inhibitors in the context of a macrophage-mediated mesangial cell scarring process using an in vitro approach.

METHODS

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich Chemical Co. (Dorset, UK).

Cell culture

Human mesangial cells were cultured, using standard serial sieving techniques, from the glomerular explants of human kidney specimens derived from the normal poles of nephrectomized kidneys from patients with renal carcinoma. The cells were cultured in RPMI 1640 (Invitrogen, Paisley, UK), supplemented with 20% heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin (Invitrogen), 100 μg/mL streptomycin (Invitrogen), 5 μg/mL bovine insulin, and 2 mmol/L glutamine (Invitrogen). Cultured cells were characterized by their stellate fusiform morphology, their positive staining for actin, myosin, vimentin, and desmin, and negative staining for factor VIII-related antigen and cytokeratin.

Mesangial cells of passages 2 through 8 were cultured in 24-well plates (Costar-Corning, Buckinghamshire, UK) or 25 cm² flasks (Costar-Corning), allowed to grow to confluence, and then rendered quiescent in RPMI medium containing 0.5% FCS for 48 hours before use.

Cells of the human monocyte/macrophage cell line U937 (ECCAC no. 85011440) were grown in RPMI 1640 supplemented with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L glutamine.

Preparation of U937 cell-conditioned medium

U937 cells were seeded into 75 cm² at a cell density of 10⁶ cells per mL in serum-free RPMI 1640 (100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L glutamine) and stimulated with 10⁻⁷ mol/L phorbol 12-myristate 13-acetate (PMA) to allow them to adhere to the plastic substrate. Adherent cells were then stimulated with lipopolysaccharide (LPS) (from Escherichia coli 026 B6) at a final concentration of 1 μg/mL in serum-free RPMI 1640 for 16 hours. After washing 3 times with Hank’s balanced salt solution (HBSS; Invitrogen), the cells were cultured for another 30 hours in serum-free RPMI 1640. The conditioned medium (MPCM) was harvested and centrifuged for 5 minutes at 1000 rpm and frozen at −20°C until required.

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 of the ACE inhibitor perindoprilat (a gift from Servier, France). The cultures were maintained in this medium for 1 or 3 days. The tissue culture supernatants were harvested and stored at −20°C for subsequent analysis.

Mesangial cells were additionally treated with MPCM ± 0.1 μmol/L bradykinin or MPCM ± ACE-1 ± 1 μmol/L bradykinin B2 receptor antagonist HOE 140.

For Northern and Southern analyses, mesangial cells were exposed to 50% MPCM ± additions for 18 hours before RNA processing.

Preparation of cell lysates

After removal of tissue culture supernatants, cell monolayers were washed with phosphate-buffered saline solution (PBS), scraped into 250 μL 1% Nonidet P40 in wash buffer (PBS containing 0.3 mol/L NaCl and 1% Tween 20), and then incubated at room temperature (RT) for approximately 30 minutes. The cell scrapings were transferred to 2 mL tubes, sonicated with a 5-second burst, and centrifuged for 1 minute at 11,600 g. The supernatants were harvested and centrifuged for 30 minutes. The tissue culture supernatants were harvested and centrifuged for another 30 minutes. The conditioned medium (MPCM) was harvested and centrifuged for 5 minutes at 1000 rpm and frozen at −20°C until required.

ELISAs

Culture supernatants and cell lysates were assayed for fibronectin using a modified version of an “in house” enzyme-linked immunosorbent assay (ELISA) [15]. Briefly, 96-well microtiter plates (Costar-Corning) were coated with 100 μL/well rabbit antihuman fibronectin antibody (in 0.05 mol/L carbonate buffer, pH 9.6) at 4°C overnight. The plates were blocked with 2% bovine serum albumin (BSA) (in wash buffer) for 1 hour at RT. After washing the plates 4 times, 50 μL/well of human fibronectin standard (2000–31.3 ng/mL in doubling dilutions), supernatant, or cell lysate were added to the wells and incubated for at least 2 hours at RT. After another 4 washes, 50 μL of monoclonal antihuman fibronectin antibody was added and incubated for 1 hour at RT. After washing, 50 μL of rabbit antimouse immunoglobulin G (IgG) conjugated to horseradish peroxidase (HRP) (Dako, Ltd., Ely, UK) was added to the wells and incubated at RT for 1 hour. After 4 more washes, 50 μL of 0.67 mg/mL 1,2 phenylenediamine dihydrochloride was added in 0.03 mol/L citrate buffer, pH 5.0, containing 0.012% H₂O₂. Following color development,
75 μL of 1 mol/L H₂SO₄ were added to stop the reaction, and absorbance was read at 492 nm on a Titertek MultiskanPlus microtiter plate reader (Flow Laboratories, Oxfordshire, UK).

The following ELISAs were carried out according to manufacturer’s instructions: (1) bradykinin enzyme immunoassay (EIA) (Peninsula Laboratories, Inc., St. Helens, Merseyside, UK); (2) BIOTRAK matrix metalloproteinase (MMP)3, MMP2, and tissue inhibitor of metalloproteinase-1 (TIMP-1) (Amersham Pharmacia Biotech, Hertfordshire, UK); (3) Quantikine MMP 9 (R&D Systems, Abingdon, Oxon, UK); (4) Quantimatrix human laminin (Chemicon International, Temecula, CA, USA); (5) Immunolysate tissue plasminogen activator (tPA) and Immunolysate plasminogen activator inhibitor-1 (PAI-1) (Biopool International, CA, USA); (6) urokinase plasminogen activator (uPA) (Oncogene Science, Bayer Diagnostics, East Walpole, MA, USA).

Zymography

MMP2 and MMP9 activities were determined using gelatin zymography. Cell supernatants were diluted 1:5 in PBS, 30 μL of the dilution was mixed with an equal volume of nonreducing sample buffer and loaded on a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel containing 1 mg/mL gelatin. After electrophoresis at 4°C, the gels were washed in 2 changes of 2.5% (v/v) Triton X-10 at RT, after which they were incubated overnight at 37°C in activation buffer (50 mmol/L Tris-HCl, 100 mmol/L NaCl, 10 mmol/L CaCl₂, 0.05% Brij 271). The gels were then stained for at least 2 hours with Brilliant Blue R, and destained in an aqueous solution methanol:acetic acid:water (40:10).

Protein determination

The protein content of cell lysates dissolved in 1% Nonidet NP40 was determined using a commercial BioRad detergent compatible (DC) protein assay, using BSA standards according to the manufacturer’s instructions.

Northern blotting

Northern analysis was carried out using a method previously described [14]. Briefly, total RNA was extracted from mesangial cells stimulated with MPCM followed by amplification using the following primers:

- Bradykinin B2 sense 5′-ATG CTC AAT GTC ACC TTG CAA-3′, antisense 5′-CTG ATG ACA CAA GCG GTG ACG-3′, tPA sense 5′-GAC TGG ACG GAG TGT GAG CTC TCC-3′, antisense 5′-GTC TAC ACC AAG GTT ACC AAC TAC-3′ [16].
- Kininogen sense 5′-GTC TAC ACC AAG GTT ACC AAC TAC-3′, antisense 5′-GTC TAC ACC AAG GTT ACC AAC TAC-3′ [16].
- Kininogen sense 5′-GTC TAC ACC AAG GTT ACC AAC TAC-3′, antisense 5′-GTC TAC ACC AAG GTT ACC AAC TAC-3′ [16].
- Kininogen sense 5′-GTC TAC ACC AAG GTT ACC AAC TAC-3′, antisense 5′-GTC TAC ACC AAG GTT ACC AAC TAC-3′ [16].
- Kininogen sense 5′-GTC TAC ACC AAG GTT ACC AAC TAC-3′, antisense 5′-GTC TAC ACC AAG GTT ACC AAC TAC-3′ [16].
- Kininogen sense 5′-GTC TAC ACC AAG GTT ACC AAC TAC-3′, antisense 5′-GTC TAC ACC AAG GTT ACC AAC TAC-3′ [16].

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Protein determination

The protein content of cell lysates dissolved in 1% Nonidet NP40 was determined using a commercial BioRad detergent compatible (DC) protein assay, using BSA standards according to the manufacturer’s instructions.

RT-PCR

Aliquots of total RNA (0.5 μg) were reverse-transcribed using avian myeloblastosis virus (AMV) reverse transcription system (Promega) 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: 1 cycle at 94°C for 5 minutes, 35 cycles of 94°C for 30 seconds, 55°C for 1 minute, 68°C for 2 minutes, 1 cycle at 68°C for 7 minutes, and 10-minute soak at 4°C.

Southern blotting

Aliquots of reverse transcription-polymerase chain reaction (RT-PCR) cDNA (20 μL) were resolved on 1% Tris-acetate EDTA (TAE)-agarose gels. The gels were denatured for 45 minutes in a solution containing 1.5 mol/L NaCl and 0.5 mol/L NaOH. The gels were rinsed in water, and then neutralized in a solution containing 1.5 mol/L NaCl and 0.5 mol/L Tris-HCl, pH 7.5. Thereafter, the gels were blotted and hybridized as described for Northern blotting.

Oligonucleotide primers

Probes were created by RT-PCR of RNA extracted from mesangial cells stimulated with MPCM followed by amplification using the following primers:

- Bradykinin B2 sense 5′-ATG CTC AAT GTC ACC TTG CAA-3′, antisense 5′-CTG ATG ACA CAA GCG GTG ACG-3′, tPA sense 5′-GAC TGG ACG GAG TGT GAG CTC TCC-3′, antisense 5′-GTC TAC ACC AAG GTT ACC AAC TAC-3′ [16].
- Kininogen sense 5′-GTC TAC ACC AAG GTT ACC AAC TAC-3′, antisense 5′-GTC TAC ACC AAG GTT ACC AAC TAC-3′ [16].
- Kininogen sense 5′-GTC TAC ACC AAG GTT ACC AAC TAC-3′, antisense 5′-GTC TAC ACC AAG GTT ACC AAC TAC-3′ [16].
- Kininogen sense 5′-GTC TAC ACC AAG GTT ACC AAC TAC-3′, antisense 5′-GTC TAC ACC AAG GTT ACC AAC TAC-3′ [16].
Perkin-Elmer Corporation, Warrington, UK) according to manufacturer’s instructions.

Metabolic labeling/immunoprecipitation

Confluent, quiescent mesangial cells were stimulated with MPCM in the presence or absence of perindoprilat or medium alone diluted 1:1 in methionine-free RPMI. Each well was pulsed with 20 μCi 35S-methionine (ICN Flow). After 3 days, the culture supernatants were retained, and the cell monolayers were washed twice in PBS. Cell lysates were prepared as described above. To immunoprecipitate the newly synthesized fibronectin, 20 μL of rabbit antihuman fibronectin antibody was added to 400 μL culture medium for approximately 4 hours. Antigen:antibody complexes were precipitated with 50 μL of insoluble protein A cell suspension (10% wet vol/vol of nonviable Staphylococcal aureus cells (Cowan strain) by overnight incubation at 4°C. The samples were centrifuged for 10 minutes at 3000g, and the pellets were washed 3 times with ice-cold immunoprecipitation buffer (PBS containing 0.5 mol/L NaCl, 0.1% SDS, and 1% Triton X-100, pH 7.4), vortexing the pellets thoroughly between each wash. The pellets were dissolved in 70 μL sample buffer and boiled for 7 minutes. The dissolved pellets were resolved by electrophoresis on 5% SDS-polyacrylamide gels. Newly synthesized fibronectin was detected by autoradiography of dried gels. Bands were quantified by scanning densitometry on a BioRad densitometer.

Bradykinin receptor radioligand-binding assays

Mesangial cells in 24-well culture plates were exposed to MPCM, MPCM ± perindoprilat, or medium alone for 18 hours. The cells were then placed on ice, and 500 μL aliquots of the culture supernatant were removed and retained. Prolyl2-4, 3,4(n)-3H-bradykinin [2 μL (0.04 μCi)] (Amersham Biosciences) was then added to the remaining 500 μL of culture medium in each well and incubated on ice in a refrigerator at 4°C for 2 hours. To determine nonspecific binding (NS), 10 μmol/L unlabeled bradykinin was added to some of the wells that had been treated with 3H-bradykinin. After the 4°C incubation, the culture supernatants were removed, and the cells were washed with cold Tris-buffered saline (TBS), pH 8.0. The cell monolayers in each well were scraped into 210 μL of 0.5 mol/L NaOH. The plates were then incubated at 65°C for 15 minutes to solubilize the protein. A portion of each cell lysate (180 μL) or 200 μL of retained radioactive culture supernatant was placed into scintillation vials containing 4 mL Ecoscint scintillation fluid (National Diagnostics, Hull, UK) and 50 μL concentrated HCl. Counts per minute (cpm) were determined on a liquid scintillation counter (LKB Wallac; Beckman Coulter, Fullerton, CA, USA). Bradykinin binding in cell lysates (cpm) as a percentage of total bradykinin input and correcting for nonspecific binding is expressed as a function of cell protein.

STATISTICS

Mesangial cell fibronectin levels were corrected for cell protein. Fibronectin levels have been expressed as fold-increase over control (medium) levels. Where appropriate, data in other experiments were expressed as percent of MPCM-stimulated levels. Representative autoradiographs of Northern or Southern blots are shown, but densitometric analysis incorporates data from all experiments after normalization for the appropriate housekeeping gene. Results are expressed as mean ± SEM. For comparison of the mean between two groups, an unpaired t test was employed. Statistical significance was defined as P < 0.05.

RESULTS

Effect of perindoprilat on mesangial cell fibronectin levels

Stimulation of confluent, quiescent mesangial cells with MPCM for 3 days resulted in a 79 ± 15% increase in secreted, and a 77 ± 17% increase in cell-associated, fibronectin levels (P < 0.001) over those of control cells (medium alone) (Fig. 1). Addition of 40 μmol/L perindoprilat significantly attenuated this response, resulting in decreases in MPCM-stimulated fibronectin levels of 19.4 ± 0.6% (P < 0.001) and 21.7 ± 1.0% (P < 0.001) for secreted and cell-associated fibronectin levels, respectively (Fig. 1). The dose of 40 μmol/L had previously been determined as optimal from a dose-response curve carried out in preliminary experiments (data not shown). Addition of 1 μmol/L LPS to mesangial cells had no effect on fibronectin production (data not shown).

Similarly, perindoprilat reduced MPCM-stimulated supernatant laminin protein levels by 23.2 ± 1.72% (P = 0.001) (data not shown).

Effect of perindoprilat on fibronectin synthesis

In order to assess whether the fibronectin levels had decreased because of a reduction in synthesis, immunoprecipitation of metabolically labeled 35S-methionine-fibronectin in the presence or absence of 40-μmol/L perindoprilat was carried out. The resulting autoradiographs demonstrated that perindoprilat did not appear to decrease fibronectin protein synthesis (Fig. 2). Moreover, this was supported by Northern blot analysis, in which fibronectin mRNA levels were unchanged in the presence of perindoprilat (Fig. 3).
Effect of perindoprilat on fibronectin synthesis

Because a decrease in fibronectin levels can also occur as a result of matrix degradation we investigated parameters which may be involved in matrix degradation. Tissue culture supernatant levels of the MMP 2, 3, and 9 were measured by ELISA. While MPCM up-regulated MMP levels over control (medium alone), total MMP protein levels were not significantly changed by treatment with perindoprilat, and neither was expression of TIMP-1 (Table 1). Gelatin zymography confirmed these data, demonstrating that there was no difference in MMP2 or 9 activities following ACE treatment of MPCM-stimulated mesangial cells (data not shown). Together these data suggest that if ACE-I induced degradation was occurring, it was being mediated by proteinases other than MMP 2, 3, and 9.

Effect of perindoprilat on fibronectin degradation

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**Table 1.** Effect of perindoprilat on supernatant levels of matrix metalloproteinases (MMPs 2, 3, 9) and tissue inhibitor of metalloproteinases (TIMP-1)

<table>
<thead>
<tr>
<th>MMP</th>
<th>ng/mL</th>
<th>MPCM</th>
<th>MPCM + ACE-I</th>
<th>Medium</th>
<th>P value vs. MPCM</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2</td>
<td>3.7 ± 0.65</td>
<td>2.7 ± 0.5</td>
<td>1.7 ± 0.4</td>
<td>0.228</td>
<td></td>
</tr>
<tr>
<td>MMP-3</td>
<td>8.1 ± 3.1</td>
<td>7.4 ± 3.2</td>
<td>0</td>
<td>0.873</td>
<td></td>
</tr>
<tr>
<td>MMP-9</td>
<td>5.5 ± 0.3</td>
<td>5.8 ± 0.5</td>
<td>0.5 ± 0.4</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>TIMP-1</td>
<td>346 ± 75</td>
<td>390 ± 69</td>
<td>220 ± 66</td>
<td>0.683</td>
<td></td>
</tr>
</tbody>
</table>

**Renin-angiotensin and kallikrein-kinin systems**

ACE activity could not be detected in our supernatants or MPCM at the sensitivity levels of an automated Sigma ACE detection assay. However, ACE gene expression was detected by semiquantitative RT-PCR, and was
found not to change in response to ACE-I treatment (data not shown). ACE provides a common link between the renin-angiotensin and the kallikrein-kinin systems—two interactive, but often antagonistic, systems involved in renal homeostasis. Inhibition of ACE activity results not only in a decrease in Ang II levels, but is accompanied by an increase in local levels of bradykinin. Semiquantitative RT-PCR demonstrated that mesangial cells not only expressed all the components of the RAS (renin, ACE and angiotensinogen) but they also expressed message for high and low kininogens and the serine protease tissue kallikrein (Fig. 4A), confirming that mesangial cells possess the biochemical machinery necessary to generate vasoactive kinins from an endogenous source.

In addition, semiquantitative RT-PCR demonstrated that MPCM stimulation reduced mesangial cell expression levels of low kininogen, the preferred bradykinin precursor in the kidney, below basal levels. Treatment with perindoprilat partially attenuated this reduction, returning levels closer to those of the control (Fig. 4B).

The role of bradykinin

We investigated the possibility that raised bradykinin levels may play a role in the observed antifibrotic effects. Bradykinin (0.1 μmol/L) was added directly to MPCM-stimulated and control mesangial cells. This resulted in a 21.5 ± 1.4% ($P < 0.02$ vs. MPCM) reduction in secreted fibronectin levels compared with those induced by MPCM alone (Fig. 5). Bradykinin also significantly decreased basal fibronectin levels by 14.1 ± 0.4% ($P < 0.001$) (Fig. 5).

Paradoxically, however, mesangial cell culture supernatant levels of bradykinin at day 3, as measured by EIA, were found to be significantly reduced following treatment with perindoprilat, and not increased as might have been expected (2.75 ± 0.64, *1.46 ± 0.23, 0.56 ± 0.08 pg bradykinin/μg cell protein for MPCM, MPCM + perindoprilat, medium alone, respectively, *$P = 0.036$ vs. MPCM) (Fig. 6).
Effect of perindopril on bradykinin receptor expression

One possible explanation for the observed decrease in bradykinin levels could be that ACE-I treatment up-regulates the expression of the bradykinin B2 receptor, with the result that more bradykinin is sequestered from the local surroundings via ligation to receptors on the cell membrane.

Semi-quantitative RT-PCR and Southern blotting of RNA from mesangial cells stimulated with MPCM demonstrated that bradykinin B2 receptor expression was indeed significantly up-regulated 71 ± 30% ($P = 0.032$) in the presence of perindopril (Fig. 7).

Moreover, bradykinin B2 receptor binding using $^3$H-bradykinin supported these observations at protein level. More $^3$H-bradykinin (71.0 ± 15.0%) bound to the surface of ACE-I–treated mesangial cells than nontreated cells ($^*P = 0.002$ vs. MPCM alone) (Fig. 8).

Effect of bradykinin-B2 receptor inhibitor HOE 140

If the antifibrotic effects of perindopril were mediated by ligation of bradykinin to the B2 receptor, treatment with a selective bradykinin B2 receptor antagonist would be expected to attenuate the beneficial effects of the ACE-I. Incubation of mesangial cells with the bradykinin B2 receptor antagonist HOE 140 (1 μmol/L) resulted in a reversal of the perindopril-mediated reduction in mesangial cell fibronectin levels (Fig. 9).

Effect of perindopril on the tissue plasminogen activator system

The plasminogen activator system is also known to be involved in extracellular matrix degradation. Bradykinin has previously been shown to modulate levels of the fibrinolytic enzyme tPA [22], while ACE-I has been shown to modulate levels of the tPA inhibitor PAI-1 [22]. Little information is available in the literature about the effects of bradykinin or ACE-Is on urokinase plasminogen activator system.
activator (uPA) activity. In order to investigate a possible link between the observed antifibrotic effects induced by perindoprilat and the expression of these agents, mRNA levels of tPA, uPA, and PAI-1 in MPCM-stimulated mesangial cells in the presence or absence of perindoprilat were examined. Northern analysis revealed a 40 ± 8.0% ($P = 0.006$) increase in tPA mRNA levels following ACE inhibitor treatment. uPA and PAI-1 mRNA levels were not significantly affected (Fig. 10).

However, measuring total tPA protein levels by ELISA demonstrated that tPA levels in the culture supernatants of ACE-I treated cells were unexpectedly, significantly, reduced by 14.0 ± 0.5% ($P < 0.001$) compared with non-ACE-I–treated cells. uPA levels were not significantly affected, while PAI-1 protein levels were reduced by 9.0 ± 0.25% ($P < 0.001$) (Fig. 11). Exogenous bradykinin treatment of MPCM-stimulated mesangial cells followed a similar pattern. Exogenous bradykinin reduced tPA levels by 25.13 ± 2.37% ($P < 0.001$), had no significant effect on uPA levels, and reduced PAI-1 levels by 18.75 ± 1.38% ($P = 0.02$) compared with nonbradykinin-treated cells (Fig. 12).

**Effect of the bradykinin B2 receptor antagonist HOE-140 on plasminogen activator system**

In order to observe the effect of bradykinin B2 receptor antagonism on components of the plasminogen activator system, supernatants from MPCM-stimulated mesangial cells treated with perindoprilat ± HOE 140 were analyzed for tPA, uPA, and PAI-1 levels by ELISA. HOE 140 treatment reversed the effects of perindoprilat on tPA and PAI-1 levels (Fig. 13), supporting the concept of a direct role of the bradykinin B2 receptor in modulating the components of the plasminogen activator system.

**DISCUSSION**

ACE-I are valuable therapeutic agents for the management of hypertension, and have specific benefits in delaying the progression of chronic renal failure. While the antihypertensive action of ACE-I may account for their renoprotective effects, other mechanisms may also be important. It is, therefore, of note that in studies in both experimental animals and humans, other classes of antihypertensive agents exhibited less renoprotection than ACE-I, despite equivalent blood pressure control. Thus, it would appear that the beneficial effects of ACE-I are mediated by mechanisms in addition to, or independent
of, their systemic hemodynamic actions. These may involve specific actions on intraglomerular hemodynamics, but also include direct effects on the cellular production of profibrotic cytokines and extracellular matrix. A large body of evidence supports the hypothesis that the effects of ACE inhibitors result from the inhibition of Ang II formation. However, accumulating data also suggest that some of their actions may be caused by reduced bradykinin catabolism, with resultant accumulation of endogenous bradykinin levels [23].

The current study has demonstrated that the antifibrotic effects of the ACE-I perindoprilat, in the context of macrophage-induced mesangial cell injury, are mediated, at least in part, by effects on the bradykinin axis. RT-PCR demonstrated that mesangial cells have the necessary biochemical machinery to generate endogenous kinins. Moreover, we demonstrated that MPCM treatment reduced basal mRNA levels of the bradykinin precursor low kininogen, which were partially restored following treatment with ACE-I. Addition of exogenous bradykinin reduced MPCM-induced fibronectin levels in mesangial cells, bradykinin B2 receptor mRNA levels were up-regulated in the presence of perindoprilat, as was binding of [H]3-bradykinin. Furthermore, inhibition of B2 receptor activity with HOE 140 attenuated the beneficial effects of perindoprilat treatment. Bradykinin exerts most of its physiologic effects via the B2 receptor, which is constitutively expressed in many cell types [24]. There is increasing experimental evidence to suggest that ACE-I may potentiate the effects of bradykinin using mechanisms that are independent of their ability to inhibit ACE activity, per se [25–29]. For example,
ACE inhibitors can potentiate bradykinin activity in the presence of ACE-resistant bradykinin B2 receptor agonists [26]. Furthermore, the bradykinin-potentiating effects of ACE-inhibitors are not mimicked by the synthetic ACE substrate hippuryl-L-histidyl-L-leucine, which is as equally effective at blocking bradykinin catabolism as are ACE-I [26, 27]. Moreover, when B2 receptors are desensitized and no longer responsive to extra agonist, ACE inhibitors can reactivate B2 receptor-mediated signaling [30]. Several possibilities may explain these phenomena. There is evidence to suggest that ACE-I may exert their effect directly on the bradykinin-B2 receptor [26, 27], although ACE-I binding to receptor is yet to be demonstrated [31]. It has also been suggested that binding of ACE-I to ACE results in a conformational change, which is transduced directly to the B2 receptor in a sort of ACE-B2 receptor “cross-talk” [30, 32]. It has been suggested that the variability in ACE-I efficacy, as seen with different molecules of the same class, may be dependent on the ACE-I’s unique structural properties, which are able to facilitate bradykinin B2 receptor signaling [25].

In the current study, bradykinin levels were significantly reduced in day 3 culture supernatants of mesangial cells treated with both MPCM and ACE-I compared with MPCM alone, results which were contrary to what might have been expected following inhibition of bradykinin catabolism. However, these observations may be explained when increased ligation to an increased number of receptors is taken into account.

The small increase in endogenous bradykinin levels over control observed in response to MPCM alone was probably insufficient to have a beneficial effect on mesangial cells comparable to that of adding exogenous bradykinin. Moreover, this slight increase is likely to be under post-transcriptional control because mRNA levels of the bradykinin precursor low kininogen in response to MPCM were lower than basal mRNA levels.

Bradykinin has been shown to be a potent stimulator of tPA in endothelial cells [33], perfused tissue preparations [34], and in whole animals [35]. A study by Brown et al [35] demonstrated that intravenous infusion of bradykinin caused a significant increase in plasma levels of tPA, which was further potentiated by treatment with ACE-I. Bradykinin appeared to have no effect on PAI-1 levels [35]. Schanstra et al demonstrated that transgenic rats overexpressing increased endogenous bradykinin exhibited reduced interstitial fibrosis in the unilateral ureteral obstruction model of renal injury. Moreover, they demonstrated that genetic manipulation or pharmacologic blockade of the bradykinin B2 receptor increased interstitial fibrosis, and was accompanied by reduced activity of extracellular matrix degrading enzymes [36].

In the present study, no measurable difference in the expression of the matrix metalloproteinases 2, 3, or 9 following ACE-I treatment could be detected. However, this was not the case for components of the plasminogen activator system. Protein expression profiles of tPA and PAI-1 were altered in response to ACE-I treatment. The protein profiles differed from mRNA expression levels, suggesting that the cells’ plasminogen activator phenotype was modulated post-transcriptionally. This pattern of plasminogen activator system expression following ACE treatment was echoed by addition of exogenous bradykinin. Moreover, antagonism of the bradykinin receptor resulted in a reversal of the effects of perindoprilat on plasminogen activator components. Together, these data lend support to the hypothesis that ACE inhibition, with its resulting reduction in extracellular matrix expression, involves bradykinin, its receptor,
and the plasminogen activator system acting in a coordinated manner.

The apparent paradoxical reduction in tPA protein levels, though unexpected, may be explained by an increased turnover of the protease via the ubiquitous low-density lipoprotein receptor-related protein (LRP) [37]. LRP is a multifunctional endocytic receptor implicated in the modulation of a number of cellular processes, including extracellular matrix accumulation and catabolism, and the turnover of proteases, and protease/inhibitor complexes. Noguchi et al have previously demonstrated that in mouse hepatocytes interleukin-1β (IL-1β) was able to up-regulate tPA mRNA levels while concomitantly down-regulating tPA activity and protein levels by up-regulating the expression of LRP [37]. A similar scenario may account for our observations. However, investigations into this phenomenon are beyond the scope of the current study.

CONCLUSION

This study provides further insight into the potential mechanisms underlying the unique renoprotective actions of ACE-I. It demonstrates the importance of bradykinin, its B2 receptor, and the plasminogen activator system in modulating the renoprotective effects of ACE-I.

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