Pharmacological Enhancement of the Kallikrein-Kinin System Promotes Anti-Fibrotic Responses in Human Mesangial Cells

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Key Words

Kallikrein-kinin system • Fibronectin • Bradykinin B2 receptor • Vasoepitidase inhibitor

Abstract

The aim of the present study was to investigate whether pharmacological enhancement of the renal kallikrein-kinin system using the vasoepitidase inhibitor omapatrilat plays a direct role in modulating the fibrotic responses of human mesangial cells to injury. Treatment with 40 μ mol/L omapatrilat was able to reduce macrophage-conditioned medium (MPCM)-induced fibronectin levels without affecting mRNA expression. MPCM injury also suppressed kallikrein and low molecular weight kininogen mRNA. Omapatrilat was able to attenuate this suppression. Bradykinin levels in contrast were increased by MPCM and treatment with omapatrilat further augmented levels. Co-incubation with the bradykinin B2 receptor antagonist HOE 140 attenuated the omapatrilat-induced lowering of fibronectin. Moreover, inhibition of cGMP release had a similar effect. Paradoxically, RT-PCR and Southern blotting demonstrated that bradykinin B2 receptor mRNA levels were down regulated in response to omapatrilat. Western blotting

supported this data. Supernatant levels of tissue plasminogen activator (tPA), a product of bradykinin stimulation, were decreased by omapatrilat while cell associated tPA levels were increased. Matrix metalloproteinase-9 (MMP-9) mRNA expression was up regulated by omapatrilat treatment, although no difference in active zymogen levels was observed. In conclusion enhancement of kallikrein-kinin system appears to play a direct role in promoting anti-fibrotic responses in MPCM-injured human mesangial cells.

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Introduction

It has been known for some time that urinary kallikrein excretion is inversely correlated with the level of blood pressure [1]. More recently it has also become apparent that urinary kallikrein levels reflect the severity of renal injury in kidney patients with chronic kidney disease [2]. Genetic manipulation of the kallikrein-kinin

system and the pharmacological administration of kallikrein in hypertensive experimental animals has not only been shown to reduce blood pressure but also to attenuate tubular and glomerular sclerotic injury [3, 4]. It is now thought that the kallikrein-kinin system can confer renoprotective effects beyond its ability to modulate renal haemodynamics. Studies have shown that long-term infusion of tissue kallikrein can attenuate glomerulosclerosis in Dahl sensitive rats without affecting their blood pressure [5]. We have previously demonstrated that the anti-fibrotic actions exhibited by the ACE-inhibitor perindoprilat on cultured human mesangial cells occur as a result of modulation of bradykinin [6] - an effector molecule of the renal kallikrein-kinin system. Moreover, addition of exogenous bradykinin to human mesangial cells was able to reproduce some of the anti-fibrotic effects of the ACE inhibitor [6]. We therefore reasoned that any agent, which is able to enhance the kallikrein-kinin system should exert beneficial effects on the kidney in the context of chronic kidney disease. More specifically we hypothesise that the kallikrein-kinin system plays a pivotal role in the modulation of fibrosis.

Vasopeptidase inhibitors, of which omapatrilat is one of the first in its class, are potent anti-hypertensives that combine ACE and neutral endopeptidase (NEP) inhibition in a single molecule formulation [7]. Like ACE, NEP is also known to catabolise bradykinin (as well as several other vasodilator molecules including the natriuretic peptides and adrenomedullin) [8]. Since both ACE and NEP are major enzymes of bradykinin catabolism in the kidney it is likely that renal bradykinin levels will be further enhanced following vasopeptidase inhibitor treatment.

While *in vivo* studies can focus on the final outcome of the beneficial effects of kallikrein-kinin system manipulation, the cellular and molecular mechanisms underlying any additional, blood pressure independent, anti-fibrotic effects are much more difficult to define. In order to investigate such mechanisms of action we have chosen to use an *in vitro* mesangial cell culture system. Mesangial cells play a pivotal role in the pathological processes in glomerulosclerosis as they contribute to the structural support of the glomerulus and are instrumental in the control of the glomerular filtration rate. In addition we have previously shown that mesangial cells possess the biochemical machinery necessary to generate vasoactive kinins from an endogenous source [6].

The aim of the present study therefore, was to extend our previous investigations on the anti-fibrotic actions of anti-hypertensive agents and examine at cellular and molecular levels the effects of injury on the mesangial

cell kallikrein-kinin system and to investigate a possible correlation between pharmacological enhancement of the renal kallikrein-kinin system with the downstream anti-fibrotic responses of mesangial cells.

Materials and Methods

Chemicals

Omapatrilat was a gift from Bristol-Myers Squibb. RPMI 1640, Hank's balanced salt solution penicillin, streptomycin, glutamine and TRIzol reagent were purchased from Invitrogen (Paisley, UK). All primers were custom made by Invitrogen. Insulin, foetal calf serum, phorbol 12-myristate acetate, lipopolysaccharide from *E. coli* 026 B6, bradykinin, kallikrein, Denhardt's solution, salmon testes DNA, formamide, Tween 20, HOE-140, PD123319, SigmaFast BCIP/NBT and DAB tablets, Kodak Xomat-AR and Biomax MS films, were purchased from Sigma (Poole, Dorset, UK). Prime-a-gene labelling system and AMV Reverse Transcription System were purchased from Promega (Southampton, UK). Reddyload mastermix was purchased from ABgene (Surrey, UK). Rabbit anti human fibronectin antibody, mouse anti-human fibronectin monoclonal antibody, fibronectin, were purchased from Merck Biosciences (Nottingham, UK). Rabbit anti-human bradykinin B2 receptor (bkb2) was purchased from Transduction Laboratories (KY, USA). Rabbit anti-mouse IgG conjugated to horseradish peroxidase and swine anti rabbit IgG conjugated to alkaline phosphatase were purchased from Dako (Ely, UK). BioRad DC protein assay was purchased from BioRad (UK). Hybond N nylon membrane was purchased from Amersham Pharmacia (Amersham, UK). Biopool Imulysse tPA and TintElize PAI-1 ELISAs were purchased from Alpha Laboratories (Hampshire, UK). Bradykinin EIA was purchased from Peninsula Laboratories, Inc., (St Helens, Merseyside, UK).

Cell culture

Human mesangial cells were cultured from the glomerular explants processed from the normal poles of nephrectomised human kidneys with renal carcinoma using standard serial sieving techniques [6]. All donors had given consent for post-surgical use of their kidneys.

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

Preparation of U937 cell conditioned medium

U937 cell conditioned medium was prepared as previously described [6].

Preparation of cell lysates

After removal of tissue culture supernatants, cell monolayers were washed with PBS, scraped into 250µl 1% Nonidet P40 in wash buffer (PBS containing 0.3mol/L NaCl and 1% Tween 20) and then incubated at room temperature for approximately 30 min. The cell scrapings were transferred to

2ml tubes, sonicated with a 5s burst and centrifuged for 1min at 11 600 x g. Sonication and centrifugation were repeated, and the lysate supernatants were assayed for total cell protein.

Culture of mesangial cells in the presence of macrophage conditioned medium (MPCM)

Confluent, quiescent mesangial cells were exposed to a 50% solution of MPCM in the presence or absence of omapatrilat. 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 exposed to MPCM ± omapatrilat were additionally treated with 1µmol/L bradykinin B2 receptor antagonist HOE 140. For Northern and Southern analyses mesangial cells were exposed to 50% MPCM ± additions for approximately 18h prior to RNA processing.

Fibronectin ELISA

Culture supernatants and cell lysates were assayed for fibronectin as previously described [6].

Protein Determination

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

Kallikrein activity assay

Relative kallikrein activity was measured in culture supernatants using a specific tissue kallikrein substrate S-2266TM (Chromogenix). Briefly 40µl supernatant was added to duplicate wells of a 96 well microtitre plate. 20µl 1.5mM S-2266 was then added followed by 50µl of 0.2mol/L Tris-HCl pH8.2. The microtitre plate was incubated at 37°C with shaking. Optical density at zero, 1h and 2h was measured. Relative kallikrein activity in each treatment was measured as the change (Δ) in optical density at 405nm.

Western blotting

Mesangial cell monolayers were scraped directly into reducing sample buffer (150µl per well) and sonicated. The samples were boiled for 5min and resolved on 8% SDS-polyacrylamide gels. The gels were blotted onto nitrocellulose membranes and immunostained. Briefly, the membranes were blocked with a 2% solution of BSA in TTBS (TBS containing 0.5% Tween 20) for 1h. The membranes were then washed once in TTBS prior to incubation with anti-human bkb2 receptor (1:1000) monoclonal antibody for at least 2h at room temperature. After 3 more washes the membranes were incubated with horseradish peroxidase labelled anti-mouse immunoglobulins for at least 2h at room temperature. The membranes were washed a further 3 times before addition of substrate (SigmaFast DAB (diamino benzidine) tablets).

Gelatin Zymography

Culture supernatants were mixed 1:1 with non-denaturing sample buffer and resolved at 4°C on 8% SDS-polyacrylamide gels containing 2.5mg/ml gelatin. The gels were washed in 2.5%

Triton X 100 for approximately one hour after which they were incubated overnight at 37°C in pH7.6 developer buffer (50mol/L Tris-HCl, 100mmol/L, NaCl, 10mmol/LM CaCl₂, 0.05% Brij) The gels were then stained with Brilliant Blue R® and destained with a solution of 40% methanol 10% acetic acid. Destained gels were dried and bands of lysis scanned using a BioRad Imaging densitometer (model GS-700).

Northern blotting

Northern analysis was carried out using a method previously described [9].

RT-PCR and Southern blotting

When messenger RNA levels were expressed at levels that could not easily be detected by Northern blotting, semi-quantitative RT-PCR and Southern blotting was performed.

RT-PCR

0.5µg aliquots of total RNA were reverse transcribed using AMV reverse transcription system according to the manufacturer's instructions. The resulting cDNA was amplified using ReddyMixTM PCR Mastermix and 50pmol of specific sense and anti-sense primers. Thermocycling conditions were optimised for each primer pair.

Southern blotting

20µl aliquots of RT-PCR cDNA were resolved on 1% TAE-agarose gels. The gels were denatured for 45min in a solution containing 1.5mol/L NaCl and 0.5mol/L NaOH. The gels were rinsed in water and then neutralised in a solution containing 1.5mol/L NaCl and 0.5mol/L Tris-HCl pH7.5. Thereafter, the gels were blotted and hybridised as described for Northern blotting. GAPDH was used as the 'house-keeping' gene to normalise for DNA loading. GAPDH did not appear to be affected by treatment with MPCM.

Oligonucleotide primers

RT-PCR was carried out on RNA extracted from mesangial cells injured with MPCM using the following primers:

Bradykinin B1 Receptor sense 5'-AGA AAC CTC CCA AGA CAG CA-3', antisense 5'-AGG AAT GTG GGG ATG CTC AA-3', Bradykinin B2 receptor sense 5'-ATG CTC AAT GTC ACC TTG CAA-3', antisense 5'-CTG ATG ACA CAA GCG GTG ACG-3', Tissue kallikrein sense 5'-AAC ACA GCC CAG TTT GT-3', antisense 5'-CTT CAC ATA AGA CAG CAC-3' [10], GAPDH sense 5'-ACG GAT TTG GTC GTA TTG GG-3', antisense 5'-TGA TTT TGG AGG GAT CTC GC-3' [11], MMP9 sense 5'-AAG TAC TGG CGA TTC TCT GAG GG-3', antisense 5'-GGC TTT CTC TCG GTA CTG GAA GAC-3' [11], MMP2 sense 5'-TTT TCT CGA ATC CAT GAT GG-3', antisense 5'-CTG TG CAG CTC TCA TAT TT-3' [12], low kininogen sense 5'-GTCACC TAA GGT CCT GCG AG-3' antisense 5'-AGG CAG AGT GAT AAA ATG GC-3' [13].

Statistics

Mesangial cell fibronectin levels were corrected for cell protein. Fibronectin has been expressed as a percentage of levels obtained following exposure to MPCM. Representative

Fig. 1. Effect of omapatrilat on mesangial cell fibronectin levels. Histogram shows the effect of treatment with omapatrilat on fibronectin levels in control and MPCM-injured mesangial cells. Results are expressed as mean $\pm$ sem as a % of MPCM-stimulated levels (* p <0.001 vs MPCM). Abbreviations: MPCM, macrophage conditioned medium; oma, omapatrilat; Med, medium alone (control).

Fig. 2. Agarose gel showing effect of omapatrilat treatment on the expression of low kininogen and kallikrein in MPCM-injured mesangial cells. Representative gels from 5-7 independent experiments are shown. Histograms show densitometric data derived from all experiments. The data for the treatment groups are expressed as a function of medium alone (control). (* p =0.002 vs MPCM, MPCM+oma vs Med =N.S., # p =0.001 vs MPCM, # # p =0.007 vs Med). Abbreviations LK1, low molecular weight kininogen; MPCM, macrophage conditioned medium; oma, omapatrilat; Med, medium alone (control).

autoradiographs of Northern or Southern blots, photographs of agarose gels or zymograms are shown but densitometric analysis incorporates data from all experiments. Results are expressed as means $\pm$ sem. For comparison of means between two groups, an unpaired t -test was employed. To compare values between multiple groups an analysis of variance (ANOVA) with Bonferroni correction was applied. Statistical significance was defined as P <0.05.

Results

MPCM-injured mesangial cell fibronectin levels

Previous studies from our laboratory have shown that MPCM increases fibronectin protein in human mesangial cells over control levels [6]. A pilot dose response curve demonstrated that 40 μ mol/L omapatrilat maximally reduced fibronectin without compromising cell

Fig. 3. Effect of omapatrilat treatment on kallikrein activity. Graph shows the effect of omapatrilat treatment on kallikrein activity measured in mesangial cell culture supernatants expressed as change in arbitrary OD₄₀₅ units. Results are means $\pm$ sem. (* $p < 0.05$ vs MPCM, $n = 6$) Abbreviations MPCM, macrophage conditioned medium; oma, omapatrilat; Med, medium alone (control).

Fig. 4. Effect of bradykinin B2 receptor antagonist HOE 140 on the fibronectin levels detected in the supernatants of MPCM-injured, omapatrilat treated mesangial cells. Results show means $\pm$ sem as a % of MPCM stimulated levels. (* $p < 0.001$ vs MPCM, no significant difference between MPCM vs MPCM + HOE140 and MPCM vs MPCM + omapatrilat + HOE140, $n = 5$ (ANOVA)). Abbreviations MPCM, macrophage conditioned medium; HOE, HOE140 (icatabant); Med, medium alone (control).

viability (data not shown). Consequently 40 μ mol/L omapatrilat was the dose chosen for all subsequent experiments. 40 μ mol/L omapatrilat reduced fibronectin levels by $39.1 \pm 6.2\%$ ($p < 0.001$ vs MPCM, $n = 8$) in MPCM-stimulated mesangial cells (Fig 1). There appeared to be no significant effect of omapatrilat on basal fibronectin levels (Fig 1).

Northern analysis demonstrated that fibronectin mRNA levels were not affected by omapatrilat treatment suggesting that the observed decrease in fibronectin was not due to reduced synthesis (data not shown).

The kallikrein-kinin system

RT-PCR followed by Southern blotting demonstrated that exposing mesangial cells to MPCM suppressed mRNA levels of the kallikrein substrate low molecular weight kininogen (LK1) to $51.6 \pm 5.4\%$ of control levels ($p = 0.001$, $n = 5$). This suppression was attenuated by omapatrilat raising mRNA levels by $76.8 \pm 13.1\%$ ($p = 0.002$ vs MPCM, MPCM + oma vs Med = NS, $n = 5$) (Fig 2). Similarly, kallikrein mRNA levels were reduced to $68 \pm 11.2\%$ of control levels ($p = 0.014$ vs medium alone, $n = 7$). Again omapatrilat was able to increase MPCM-reduced kallikrein mRNA levels by $146 \pm 18\%$ ($p < 0.001$ vs MPCM, $n = 7$) (Fig 2).

Culture supernatants were analysed for tissue kallikrein activity. MPCM-injured mesangial cells exhibited less enzyme activity than control cells. Treatment with omapatrilat increased activity to levels comparable with those of control ($p < 0.05$ vs MPCM, $n = 6$) (Fig 3).

Bradykinin levels were also affected by omapatrilat treatment. However, whereas omapatrilat upregulated MPCM-suppressed low molecular weight kininogen and kallikrein genes, omapatrilat further increased bradykinin levels that had already been augmented by prior exposure to MPCM (MPCM: 0.151 ± 0.008 , MPCM + oma: 0.216 ± 0.02 , med: 0.052 ± 0.005 ng/ml * $p = 0.02$ vs MPCM, $n = 7$).

Bradykinin B1 and B2 receptor expression

To assess the role played by the bradykinin B2 receptor in the regulation of mesangial cell fibronectin levels, MPCM-injured, omapatrilat treated cells were co-incubated with the specific bradykinin-B2 receptor antagonist HOE 140 (1 μ mol/L). Under these conditions the ability of omapatrilat to reduce fibronectin was attenuated (Fig 4). In further support of bradykinin B2 receptors being involved in our observations we found that cyclic GMP, a second messenger molecule involved in bradykinin B2 receptor activity, was significantly

Fig. 5. Effect of guanylyl cyclase inhibitor (cGMP-I) NS 2028 on fibronectin levels in the supernatants of MPCM-stimulated, omapatrilat treated mesangial cells. Results show means $\pm$ SEM as % of MPCM-injured levels. (* $p < 0.001$ vs MPCM, no significant difference between MPCM vs MPCM + omapatrilat + cGMP-I, $n = 4$ (ANOVA)). Abbreviations cGMP-I, guanylyl cyclase inhibitor; MPCM, macrophage conditioned medium; Med, medium alone (control); oma, omapatrilat.

increased by 87.7% following omapatrilat treatment (0.72 ± 0.1 , 1.34 ± 0.21 and 0.64 ± 0.16 fmol cGMP/ μ g cell protein for MPCM, MPCM + omapatrilat and medium respectively, * $p < 0.04$ vs MPCM alone, $n = 5$). Moreover, co-incubation in the presence or absence of the NO-sensitive, soluble guanylyl cyclase inhibitor NS2028 demonstrated that the fibronectin lowering effects of omapatrilat were partially attenuated (Fig 5). However, RT-PCR and Southern blotting unexpectedly demonstrated that expression of both bradykinin B1 and B2 receptor mRNAs was reduced following omapatrilat treatment. Bradykinin-B1 receptor mRNA expression was reduced by $30.7 \pm 0.17\%$, ($p = 0.02$ vs MPCM alone, $n = 8$) while bradykinin-B2 receptor mRNA expression was reduced by $38.7 \pm 5.7\%$ ($p < 0.001$ vs MPCM alone $n = 10$) (Fig 6A). Western blotting supported this data demonstrating that there was a reduction in bradykinin B2 receptor protein in the cell lysates of omapatrilat treated cells (Fig 6B).

Matrix degradation -the role of the plasminogen activator system

Bradykinin is known to be a potent stimulator of tissue plasminogen activator (tPA) release in perfused tissues [14] and endothelial cells [15]. We therefore

Fig. 6. A Southern blot showing effect of omapatrilat on MPCM-stimulated mesangial cell bradykinin B1 and B2 receptor mRNA expression. A representative autoradiograph from 10 independent experiments is shown. B Western blot showing the effect of omapatrilat treatment on the expression of bradykinin B2 receptor protein in the cell lysates of mesangial cells injured with MPCM. Abbreviations MPCM, macrophage conditioned medium; oma, omapatrilat; Med, medium alone (control).

measured protein levels of this protease and its inhibitor PAI-1 (plasminogen activator inhibitor-1) in omapatrilat-treated mesangial cells. Paradoxically omapatrilat appeared to down-regulate supernatant tPA protein levels by approximately 20% (Fig 7) while PAI-1 levels appeared to be unchanged (372 ± 50 , 386 ± 55 and 200 ± 39 ng/ml for MPCM, MPCM + oma and medium respectively).

Reduced supernatant tPA levels may be explained by an increased binding to cell surface receptors and/or increased turnover or clearance of the protease. We therefore analysed mesangial cell lysates to see if we could detect any cell associated tPA which may have bound or been internalised by the cells. The data

Fig. 7. Effect of omapatrilat on tPA protein levels in culture supernatants and cell lysates of mesangial cells injured with MPCM and treated with omapatrilat. Results show mean $\pm$ sem of ng tPA/ μ g cell protein (* $p=0.03$, ** $p<0.05$, $n=4-5$). Abbreviations MPCM, macrophage conditioned medium; oma, omapatrilat; Med, medium alone (control).

demonstrated that increased levels of tPA were detected in the cell lysates from cells treated with omapatrilat (Fig 7).

Matrix metalloproteinases

Low-density lipoprotein receptor-related protein (LRP) is a multifunctional receptor known to be involved in tPA clearance [16]. Moreover, tPA has been shown to act as a 'cytokine' via the same receptor to mediate the transcriptional regulation of matrix metalloproteinase type 9 (MMP9) [17]. We therefore sought to determine whether there was a connection between the observed

Fig. 8. A RT-PCR agarose gel showing effect of omapatrilat on MMP9 and MMP2 mRNA levels in mesangial cells following injury with MPCM and treated with omapatrilat. B Gelatin zymogram showing effect of omapatrilat on MMP9 and MMP2 activity in the supernatants of mesangial cells following injury with MPCM and treatment with omapatrilat. A representative zymogram from 8 experiments is shown.

omapatrilat-induced modulation of tPA expression and a change in MMP9 expression which could account for the observed changes in fibronectin levels following omapatrilat treatment. RT-PCR demonstrated that MMP9 mRNA levels were up-regulated in omapatrilat treated cells (0.53 ± 0.03 vs $*0.86 \pm 0.06$ for MPCM and MPCM + oma respectively, $*p=0.007$ vs MPCM, $n=4$) while MMP2 mRNA levels remained unchanged (Fig 8a). However no difference in active zymogen activity (MMP2 or MMP9) could be observed following treatment with omapatrilat by gelatin zymography (Fig 8b).

Discussion

Manipulation of the renal kallikrein-kinin system in hypertensive, experimental animals has proved protective on their renal function attenuating injury-associated glomerulosclerotic lesions and tubular injury [3, 4]. Although the precise mechanisms underlying the specific anti-fibrotic effects in response to kallikrein manipulation are not yet fully understood recent studies have shed valuable light on possible mechanisms. In gene transfer studies *in vivo* kallikrein has been shown to attenuate renal fibrosis by inhibiting oxidative stress and inflammation [18, 19]. In order to more clearly delineate the anti-fibrotic sequelae of manipulating the kallikrein-kinin system independently of the confounding effects of haemodynamic changes we employed an *in vitro* cell culture model and utilised the vasopeptidase inhibitor omapatrilat as a tool for pharmacologically enhancing the intrinsic kallikrein-kinin system.

Treatment of MPCM-injured cells with omapatrilat was able to reduce the accumulation of fibronectin in the culture medium and concomitantly reverse the suppression of kallikrein-kinin system components. As expected culture supernatant bradykinin levels were raised following treatment with omapatrilat consistent with a reduced rate of bradykinin catabolism. Blockade of the bradykinin B2 receptor or inhibition of the release of cGMP, the second messenger molecule associated with receptor activation, was able to attenuate the fibronectin lowering effects of omapatrilat. These data thus confirm an important role for the bradykinin B2 receptor in controlling fibrosis and are consistent with *in vivo* observations where regression of glomerular injury by kallikrein infusion has been proved to be a bradykinin B2 receptor mediated event [4] and where manipulation of bradykinin B2 receptor expression has been shown to have profound effects on interstitial fibrosis [20]. In addition, the fact that inhibition of cGMP release could reduce fibronectin is in accord with other reports [21] demonstrating that guanylate cyclase represents an important, and at least partially blood pressure independent anti-fibrotic pathway in glomerular disease.

Contrary to the findings reported in our previous study with the ACE-inhibitor perindoprilat, wherein bradykinin B1 and bradykinin B2 receptor mRNA expression were up-regulated and culture supernatant bradykinin levels were down-regulated [6], in the current study omapatrilat down-regulated the expression of kinin receptor mRNA and protein and increased culture supernatant bradykinin levels when analysed at the same time point. It is very

likely that kinin receptor mRNA down regulation occurs as a result of over stimulation as a direct consequence of reduced bradykinin catabolism, induced by both ACE and NEP inhibitors, and compounded by potentiation of the actions of bradykinin on the B2 receptors, that also occurs as a result of ACE inhibition. The fact that HOE 140 was able to reverse the effects of omapatrilat treatment when kinin receptor mRNA expression was suppressed would appear paradoxical. However, there is some evidence to suggest that a preformed bradykinin B2 receptor reserve may 'cushion' transcriptional modulation by some treatments [22]. This is to some extent supported by our data since the pattern of bradykinin B2 receptor protein expression did not exactly reflect mRNA levels with more receptor being present in control cells than would be suggested by the Southern blot. Similarly, the omapatrilat induced decrease in receptor protein is not as large as seen at molecular level. In contrast to the findings in this study other investigators have reported that continuous exposure of cells to bradykinin results in translational and post-translational control of kinin receptor expression with mRNA levels remaining constant [23]. Although this study has focused on the effects of the bradykinin B2 receptor, bradykinin B1 receptors may also play a role in modulating the effects of the kallikrein-kinin system. In this study B1 receptor mRNA expression exhibited a pattern similar to that of the B2 receptor, being up-regulated by MPCM and down regulated by omapatrilat. Bradykinin B1 receptors are normally expressed under conditions of inflammation and this was confirmed in our system by the fact that B1 receptor mRNA was not seen to be constitutively expressed but expressed *de novo* on treatment with MPCM. The potential role of B1 receptors in attenuating the fibrotic process has been alluded to in a study by Hagiwara et al in which they demonstrated that treatment of spontaneously hypertensive, stroke prone rats with the B1 antagonist Des-Arg9-Leu-bradykinin resulted in increases in interstitial and glomerular fibrosis, as well as collagen III and TGF β expression in [24]. A more detailed investigation into the anti-fibrotic role of B1 receptors in the actions of the kallikrein-kinin system is warranted, although it is beyond the scope of the current study.

The anti-fibrotic effects observed in response to kallikrein-kinin system enhancement in this study most probably occur via matrix degradation. Bradykinin is known to stimulate the release of tPA. Although we observed a decrease in supernatant tPA levels this was accompanied by an increase in 'cell associated' tPA levels lending support to the idea that omapatrilat was able to

induce an increase in tPA binding/clearance. An effect, which could be mediated via a receptor such as LRP, the large multifunctional, endocytic receptor involved in, amongst other things, the scavenging and recycling of proteases, as well as cell signalling [16, 17]. We observed an increase in MMP9 mRNA levels consistent with reports stating that tPA can up-regulate the transcription of MMP9 however, this did not appear to be translated into active zymogen as seen by gelatin zymography. The possible role of LRP in the anti-fibrotic actions of kallikrein-kinin system-enhancing drugs is worthy of more detailed investigation, which is beyond the scope of this study.

The differences between the effects of omapatrilat and those we have previously observed with the ACE inhibitor perindoprilat may be explained by the additional bradykinin potentiating effects of the NEP-I component of the vasopeptidase inhibitor. Our data suggest that omapatrilat is directly able to enhance the injury-suppressed components of the kallikrein-kinin system. To our knowledge this is the first study to show that the kallikrein-kinin system can play a direct anti-fibrotic role in reducing the injurious effects of MPCM exposure in human mesangial cells possibly by potentiating the

expression of extra cellular matrix degrading enzymes.

In conclusion this study lends strong support to our hypothesis that one of the renoprotective properties of the kallikrein-kinin system includes an inherent ability to promote anti-fibrotic responses - at least in mesangial cells.

Abbreviations

MMP (matrix metalloproteinase); MPCM (macrophage-conditioned medium); oma (omapatrilat); Med (medium alone control).

Acknowledgements

This study was funded, in part, by an unrestricted grant from Bristol Myers Squibb who also provided the vasopeptidase inhibitor omapatrilat. A part of this study was presented in abstract form at the annual American Society of Nephrology meeting in Philadelphia, 2002.

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