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Thrombin-Mediated Down-regulation of Endothelin Receptors in Mesangial Cells Coincides with the Down-regulation of Neutral Endopeptidase Activity

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SUMMARY

Thrombin-mediated down-regulation of endothelin (ET) receptors was studied in rat glomerular mesangial cells. Overnight incubation of mesangial cells with thrombin (10 nM) resulted in a significant decrease (67%) in the number of ET receptors, with no change in affinity. Northern analysis of the mRNA from these cells showed a corresponding decrease in the ET_A receptor message. Such a decrease in ET receptors could result from an increase in ET levels caused by an increase in synthesis and/or a decrease in degradation. It has been previously reported that thrombin stimulates ET production in endothelial and mesangial cells. Because ET is known to be degraded by neutral endopeptidase (NEP), which is present at high levels in the kidney, the potential effects of thrombin on NEP activity were evaluated. There was a decrease of NEP activity in mesangial cells at 16 and 24 hr after treatment with 10 nM thrombin. This effect was specific for thrombin, because NEP activity was not altered after treatment with thrombin in the presence of hirudin, an inhibitor of thrombin activity. The thrombin-mediated decrease in NEP activity correlated with a decrease in NEP protein and mRNA levels, as determined by Western and Northern analyses, respectively. To determine whether the thrombin-mediated decrease in ET receptors had a functional corollary, ET-1-stimulated intracellular calcium mobilization was measured. Overnight incubation with 10 nM thrombin resulted in a significant inhibition of ET-stimulated intracellular calcium mobilization. This effect was specific for ET, because thrombin pretreatment did not affect vasopressin-stimulated intracellular calcium mobilization in mesangial cells. These results indicate that the thrombin-mediated down-regulation of ET receptors is due, in part, to a thrombin-stimulated increase in ET resulting from the down-regulation of NEP and the reported increase in ET synthesis. In addition, pretreatment of mesangial cells with ET-1 caused a significant decrease (85%) in ET receptor number and ET-1-mediated intracellular calcium release (84%), without affecting vasopressin- or thrombin-mediated responses.

Thrombin, a serine protease that plays a key role in the coagulation cascade, also acts at specific receptors on a variety of cells, including mesangial cells. We have previously shown that thrombin stimulates proliferation, contraction, prostaglandin E_2 production, and intracellular calcium mobilization in mesangial cells (1). Thrombin has also been shown to increase ET-1 mRNA (2) and immunoreactive ET-1 in mesangial cells (3, 4).

ET is a potent vasoconstrictor peptide originally isolated and purified from the supernatant of cultured porcine aortic endothelial cells stimulated with either thrombin or transforming growth factor-β (5). The physiological effects of ET are mediated by its interaction with specific cell surface receptors. ET receptors have been identified in a variety of tissues and cell lines, including glomerular mesangial cells. In mesangial cells, the interaction of ET-1 with its receptor has been reported to stimulate proliferation (6–8), contraction (8–10), intracellular calcium mobilization, phosphoinositide turnover (6, 8, 11), platelet-derived growth factor secretion (7), and prostaglandin E_2 production (9).

Although it is clear that thrombin causes an increase in synthesis of ET, its effects on ET receptors and ET metabo-

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ABBREVIATIONS: ET, endothelin; NEP, neutral endopeptidase; $[Ca^{2+}]_i$, intracellular calcium concentration; S6c, sarafotoxin 6c; MES, 2-(*N*-morpholino)ethanesulfonic acid; AM, acetoxymethyl ester; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; TBST, Tris-buffered saline/Tween; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

lism have not been evaluated. Accordingly, experiments were designed to assess the effect of thrombin on ET receptor binding and function and on the activity of NEP, a proteolytic enzyme that has been shown to degrade peptide hormones such as ET, atrial natriuretic factor, and enkephalins.

Experimental Procedures

Materials. Thrombin (from human plasma), hirudin (leech, recombinant, Lys^{47} variant), insulin, glutaryl-Ala-Ala-Phe-4-methoxy-2-naphthylamine, microsomal leucine aminopeptidase, phenylmethylsulfonyl fluoride, and Fast Garnet GBC (CI 37210) were purchased from Sigma Chemical Co. (St. Louis, MO); collagenase was from Worthington Biochemical Corp. (Freehold, NJ); RPMI 1640 medium, penicillin, and streptomycin were from GIBCO Laboratories (Grand Island, NY); fetal bovine serum was from Hyclone Laboratories (Logan UT); ET-1 and S6c were from American Peptides (Sunnyvale, CA); BQ123 was synthesized at SmithKline Beecham (King of Prussia, PA); ^{125}I-ET-1 (specific activity, 2200 Ci/mmol) was from New England Nuclear (Boston, MA); and ^{125}I-Protein A (specific activity, 46 mCi/mg) was from ICN (Irvine, CA). All other chemicals were of the highest grade available.

Mesangial cell culture. Mesangial cell cultures were established from glomeruli obtained from the kidney cortex of 55–70-g rats (Sprague-Dawley; Charles River). Glomeruli were isolated by sequential sieving, which removes tubules (300- and 150-μm sieves) and then retains glomeruli on a 63-μm sieve. Isolated glomeruli were incubated for 10 min at 37° with collagenase (750 units/ml) and then plated in flasks in RPMI 1640 medium supplemented with 0.6 unit/ml insulin, 100 units/ml penicillin, 100 μg/ml streptomycin, and 15% fetal bovine serum. Cells were grown at 37° in 5% CO_2, and the cells were subcultured by rinsing with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and then incubation with 0.05% trypsin supplemented with 20 mM EDTA. The cells were identified as mesangial cells by the following criteria: 1) a stellate morphology by phase-contrast microscopy, 2) microfilaments and subplasmalemmal cytoplasmic densities by transmission electron microscopy, 3) insensitivity to puromycin aminonucleoside, and 4) positive immunofluorescence staining for actin and desmin, with negative staining for keratin and factor VIII antigens.

Measurement of $[Ca^{2+}]_i$. Intracellular calcium mobilization in response to ET and vasopressin was determined by measuring the fluorescence of fura-2-containing mesangial cells with a dual-wavelength spectrofluorometer (University of Pennsylvania Biomedical Instruments Group). Mesangial cells grown on glass coverslips (treated with 5 μg/ml poly-L-lysine) were placed in RPMI 1640 medium without fetal bovine serum, in the presence or absence of 10 nM thrombin or 100 nM ET, for 24 hr before the experiment. Just before the experiment, the cells were washed twice with Krebs buffer (118 mM NaCl, 4.6 mM KCl, 24.9 mM $NaHCO_3$, 1.0 mM KH_2PO_4, 11.1 mM glucose, 1.1 mM $MgSO_4$, 1.0 mM $CaCl_2$, 5 mM HEPES buffer, pH 7.4, 0.1% BSA). Mesangial cells were then incubated for 30 min with 2 μM fura-2/AM at 37°, washed with Krebs buffer, incubated for 20 min at 37° without fura-2/AM, and kept on ice until use. The coverslips were then placed diagonally in a square cuvette containing Krebs buffer, so that the excitation and emission paths were at 45° angles to the monolayer of cells. Intracellular fura-2/AM was excited at 340 and 380 nm and fluorescence emission was read at 510 nm, to assess bound and free fura-2/AM, respectively. The use of the 340-nm/380-nm fluorescence ratio (R) permitted normalization of the signal without regard to cell number and amount of fura-2/AM loaded into the cells. Fluorescence was recorded for 1 min, to ensure a stable base-line, before the addition of various concentrations of thrombin or vasopressin. The $[Ca^{2+}]_i$ was calculated using the following formula, as described previously (12): $[Ca^{2+}]_i = [(R - R_{min})/(R_{max} - R)] \times \beta \times 224$ (nM), where R is the ratio of fluorescence measured at 340 and 380 nm. R_{min} and R_{max} were determined in a cuvette, without cells, in Krebs buffer without Ca^{2+} plus 1 mM EGTA plus 300 nM fura 2/AM, pH 7.4 (R_{min}), or in Krebs buffer without Ca^{2+} plus 1 mM EGTA plus 2 mM $CaCl_2$ plus 300 nM fura 2/AM, pH 7.4 (R_{max}); β is the minimum fluorescence at 380 nm (R_{min}buffer)/maximum fluorescence at 380 nm R_{max}buffer.

Preparation of mesangial cell membranes. Mesangial cells grown to confluence in 150-cm^2 cell culture flasks (Corning, Corning, NY) were treated for 24 hr with reagents as indicated in the text or figure legends. The cells were washed twice with cold phosphate-buffered saline, scraped on ice, and pelleted by centrifugation at 1000 × g at 4°. The cell pellet was dispersed in a buffer containing 5 mM Tris, pH 7.5, 2 mM $MgCl_2$, 1 mM EGTA, 1.0 μg/ml leupeptin, 100 μg/ml phenylmethylsulfonyl fluoride, and 2 μg/ml aprotinin, and the mixture was then sonicated on ice for four 15-sec periods, using a Heat Systems ultrasonic processor. The lysed cell suspension was centrifuged for 10 min at 4° at 1000 × g. The supernatant was centrifuged at 40,000 × g at 4° for 30 min. The membrane pellet was resuspended in a small volume of buffer containing 50 mM Tris, pH 7.5, and 10 mM $MgCl_2$ and was then frozen in liquid nitrogen.

^{125}I-ET-1 binding. Membrane protein (3–5 μg) was added to tubes containing either buffer (50 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, 0.05% BSA) or buffer plus 1 μM unlabeled ET-1 (nonspecific binding). The reaction was started by the addition of 0.3 nM ^{125}I-ET-1, incubated for 60 min at 30°, and stopped with 3 ml of cold buffer containing 50 mM Tris·HCl, pH 7.5, and 10 mM $MgCl_2$. Membrane-bound radioactivity was separated from free ligand by filtering through Whatman GF/C filter paper that had been presoaked in 0.1% BSA. The filters were washed five times with 3 ml of buffer, using a Brandel cell harvester. Filter papers were counted in a γ counter with an efficiency of 75%. Nonspecific binding was 5–20% of total binding, depending on the concentration of ^{125}I-ET-1 used. For saturation binding experiments, increasing concentrations of ^{125}I-ET-1 (10–600 pM) were added to membranes in the absence (total binding) or presence (nonspecific binding) of 1 μM unlabeled ET-1. The data presented are from one experiment, which is representative of three or four experiments.

NEP assay. NEP assays were performed according to the methods of Hersh and Morihara (13) and Dickinson *et al.* (14), with the following modifications. Mesangial cell membranes were diluted in 100 mM MES, pH 6.5, containing 300 mM NaCl, to give a protein concentration of 0.2 μg/60 μl. The assays were carried out in 96-well microtiter plates and initiated by the addition of 20 μl of 1 mM glutaryl-Ala-Ala-Phe-4-methoxy-2-naphthylamine to the membrane preparations. The reaction was allowed to proceed for 60 min at 37°. The product, Phe-4-methoxy-2-naphthylamine, was further hydrolyzed to 4-methoxy-2-naphthylamine by the addition of 20 μl of 10 μg/ml microsomal leucine aminopeptidase (EC 34.11.2) in the presence of 2.5 μM phosphoramidon. This reaction was also allowed to proceed for 60 min at 37°. The reaction was terminated by the addition of 10 μl of 10% trichloroacetic acid. After the addition of 150 μl of 0.05% Fast Garnet GBC (CI 37210) and incubation for 30 min at room temperature, 4-methoxy-2-naphthylamine was measured at 570 nm using a Molecular Devices plate reader. A standard curve using 4-methoxy-2-naphthylamine was generated for each experiment.

Western blot analysis. Mesangial cell membranes (5 μg) were subjected to SDS-polyacrylamide gel electrophoresis (1.5-mm-thick, 4% stacking gel and 10% resolving gel) and transferred to nitrocellulose paper by electroblotting in 50 mM 3-(cyclohexylamino)-1-propanesulfonic acid, pH 11, at 300 mA overnight. Blots were blocked with 1% BSA in TBST (20 mM Tris·HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20) and probed with anti-NEP antibody (15) at a dilution of 1/2000 in TBST. After three 10-min washes in TBST, the blots were incubated with ^{125}I-Protein A (1 μCi/ml, in TBST) for 1 hr at room temperature. The blots were then washed, air dried, and autoradio-

graphed at −70° with an intensifying screen. Purified NEP was used as an internal standard (16).

Isolation of RNA. Total RNA was isolated from mesangial cells according to a modification of the procedure of Chomczynski and Sacchi (17). Briefly, mesangial cells (~10^7 cells) were lysed directly in the culture dish by the addition of RNAzol B (Biotecx Laboratories, Houston, TX). Chloroform (0.1 volume) was added to the cell lysate, which was then chilled on ice for 15 min. The phases were separated by centrifugation at 12,000 × *g* for 15 min. The aqueous phase was transferred to a fresh tube, an equal volume of isopropranol was added, and the RNA was precipitated by placing the tubes on ice for 45 min. The tubes were then centrifuged at 12,000 × *g* for 15 min. The pellets were washed once with 70% ethanol, briefly dried under vacuum, and resuspended in 0.5 ml of proteinase K buffer (0.1 M Tris·HCl, pH 7.5, 1% SDS, 50 mM NaCl, 10 mM EDTA). Proteinase K (Boehringer Mannheim, Indianapolis, IN) was added at a final concentration of 100 μg/ml, and the mixtures were incubated at 37° for 30 min and then extracted twice with phenol/chloroform/isoamyl alcohol (10:10:1). Total RNA was precipitated with 2 volumes of ethanol and 0.1 volume of 3 M sodium acetate, pH 5.2. The precipitate was collected by centrifugation, washed with 70% ethanol, and briefly dried under vacuum. For the analysis of NEP, the RNA precipitate was dissolved in 0.2 ml of TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA). For analysis of the ET_A receptor, poly(A)$^+$ RNA was isolated by chromatography on an oligo(dT)-cellulose column (Boehringer Mannheim, Indianapolis, IN) (18).

Northern analysis. For Northern analysis, 10 μg of total RNA (for NEP) or 10 μg of poly(A)$^+$ RNA (for ET_A receptor) were isolated from mesangial cells, fractionated on a 1% agarose-formaldehyde gel (5), and transferred to a UV-Duralon nylon membrane (Stratagene, La Jolla, CA). Nylon blots were prehybridized at 42° in 10 ml of 35% formamide, 5× SSPE (50 mM $NaH_2PO_4 \cdot H_2O$, 0.75 M NaCl, 5 mM EDTA, pH 7.4), 0.2% sodium pyrophosphate, 0.5% SDS, 8% dextran sulfate, 250 μg/ml salmon sperm DNA. Next, [^{32}P]dCTP-labeled (with random primers) (19) NEP (20), ET_A receptor, or β-actin cDNAs (specific activity, 2 × 10^9 cpm/μg of DNA) were added as probes and hybridized at 42° for 18 hr. The blots were washed twice with 6× SSC (0.9 M NaCl, 90 mM sodium citrate, pH 7)/0.1% SDS, followed by two washes with 2× SSC/0.1% SDS and then two washes with 0.2× SSC/0.1% SDS at 65°. The blots were then exposed to Kodak XAR-5 film, with intensifying screens, at −70°. The band densities were quantitated by scanning densitometry. For the analysis of NEP RNA, equivalent loading of RNA samples was confirmed by comparison with the 28 S ribosomal band stained with ethidium bromide. In the analysis of ET_A receptor RNA, equivalent loading of the samples was determined by comparison with β-actin mRNA.

Statistics. Statistical analyses were done using the Student *t* test and the Fisher protected least-significant difference test, to protect against making a false-positive error. Individual EC_{50} values were determined from a plot of the percentage of maximum effect versus log concentration.

Results

The effect of thrombin on ET binding was measured in membranes prepared from mesangial cells that had been treated for 24 hr with 10 nM thrombin in the presence or absence of 30 nM hirudin, a specific inhibitor of thrombin activity. Thrombin pretreatment caused a significant decrease in the total ^{125}I-ET-1 binding, relative to untreated control mesangial cells (Fig. 1A). Hirudin, which had no effect on ET-1 binding, completely abolished the thrombin-mediated decrease in ^{125}I-ET-1 binding. The effect of thrombin on ^{125}I-ET-1 binding was time dependent. The maximum inhibition occurred after 16 hr of treatment (Fig. 1B). Saturation binding isotherms for ^{125}I-ET-1 binding to membranes prepared from rat mesangial cells either without or with a

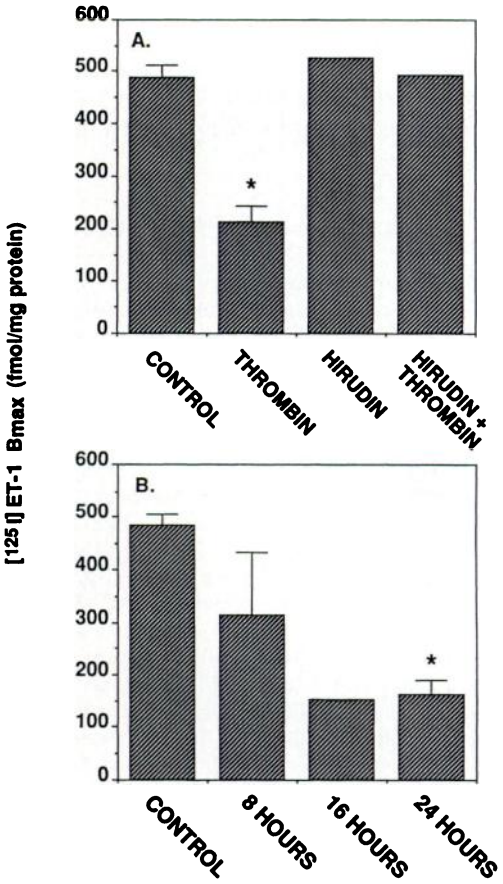
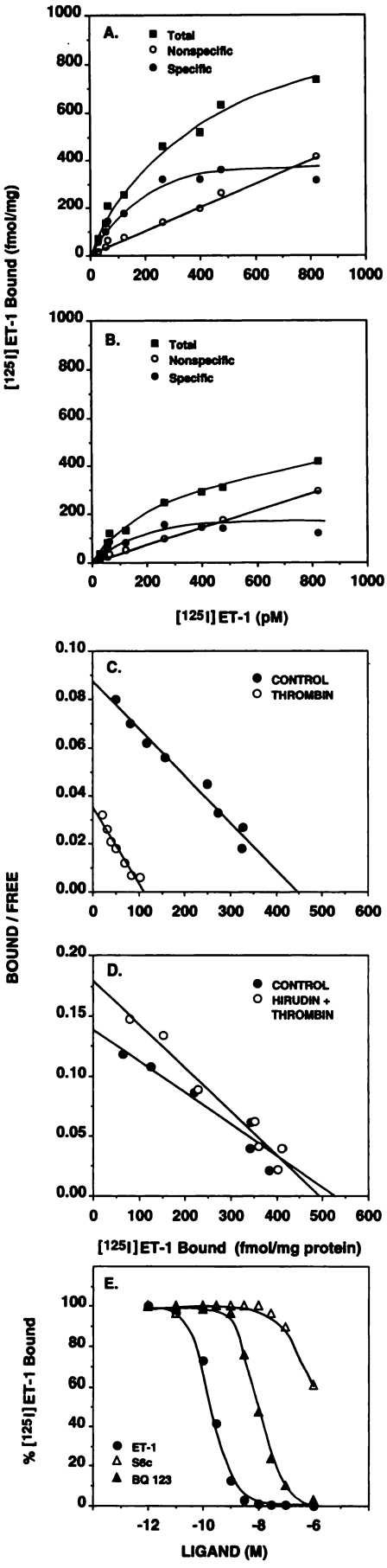


Fig. 1. Effect of thrombin pretreatment on ^{125}I-ET-1 binding to mesangial cell membranes. A, The B_{max} for ET-1 binding was determined from ^{125}I-ET-1 saturation binding experiments using membranes prepared from untreated mesangial cells (three experiments) or cells treated for 24 hr with 10 nM thrombin (three experiments), 30 nM hirudin, or thrombin and hirudin (one experiment), as described in Experimental Procedures. B, Pretreatment with thrombin caused a time-dependent decrease in the B_{max} for ET-1 binding. For each time point, three separate experiments were performed, except for the 16-hr incubation, which was done once. *, $p < 0.05$, compared with control.

24-hr thrombin pretreatment are shown in Fig. 2, A and B. The decrease in ^{125}I- ET-1 binding in thrombin-treated cells was due to a decrease in total binding rather than an increase in nonspecific binding. Scatchard analysis (Fig. 2C) of the specific binding data from Fig. 2, A and B, indicated that thrombin pretreatment decreased total ET receptors in mesangial cells from 484 ± 23 to 161 ± 29 fmol/mg of protein but had no significant effect on the dissociation constants for ^{125}I-ET-1 (89 ± 7 versus 67 ± 12 pM). Scatchard analysis of ^{125}I-ET-1 binding to membranes prepared from mesangial cells that had been pretreated with thrombin plus hirudin showed that hirudin prevented the thrombin-mediated decrease in the density of ET receptors (Fig. 2D). Competition binding experiments were performed in membranes prepared from mesangial cells, to identify the subtype of ET receptors by using the ET_A-selective antagonist BQ123 and the ET_B-selective agonist S6c (Fig. 2E). The data indicated that BQ123 was >100 times more potent (IC_{50} = 10 nM) than S6c (IC_{50} > 1 μM) in competing for the binding. This suggests that the ET receptors are predominantly of the ET_A subtype. To determine whether a decrease in ET_A receptor mRNA correlated with the decrease in the density of ET receptors,



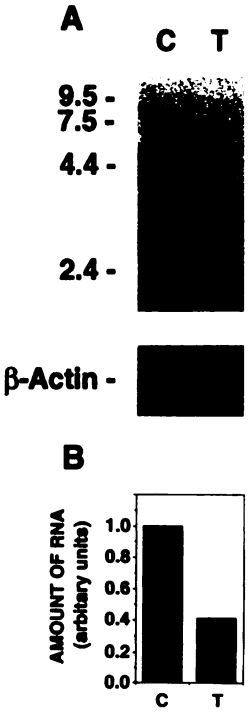


Fig. 3. A, Northern analysis of ET_A receptor mRNA from control and thrombin-treated mesangial cells is shown. Steady state levels of mRNA were determined by Northern blotting, as described in Experimental Procedures, using ET_A receptor and β-actin cDNAs from control (*C*) and thrombin-treated (*T*) mesangial cells. B, The bands corresponding to ET_A receptors in RNA were analyzed by densitometry and the ET_A receptor transcript levels were normalized to the β-actin transcript levels. The ratio of ET_A receptor to β-actin message for control cells was arbitrarily assigned a value of 1.

ET_A receptor mRNA levels were measured in untreated cells and cells treated with 10 nM thrombin. A Northern blot analysis indicated that thrombin pretreatment caused a decrease in ET_A receptor message, compared with control cells (Fig. 3A). The data were normalized to the amount of β-actin mRNA loaded onto the gel by using densitometric scanning and determining ratios of ET_A receptor mRNA transcript levels to β-actin mRNA levels. The ratio obtained from the control cells was assigned the value of 1. Mesangial cells pretreated with thrombin had a 60% decrease in ET_A receptor mRNA transcripts, compared with control cells (Fig. 3B).

To determine whether the thrombin-mediated decrease in the number of ET receptors had an effect on ET function,

Fig. 2. A and B, Saturation binding of ^{125}I-ET-1 to membranes prepared from untreated rat mesangial cells (A) or cells treated for 24 hr with 10 nM thrombin (B). Increasing concentrations of ^{125}I-ET-1 (10–820 pM) were added to membranes in the absence (total binding) or presence (nonspecific binding) of 1 μM unlabeled ET-1, and membranes were incubated for 60 min at 30°. C, Scatchard transformation of the specific binding of ^{125}I-ET-1 from A and B. D, Scatchard analysis of ^{125}I-ET-1 binding to membranes prepared from untreated mesangial cells or cells treated for 24 hr with 10 nM thrombin plus 30 nM hirudin. E, Competition by unlabeled ET-1, BQ123, and S6c with ^{125}I-ET-1 binding to membranes prepared from untreated mesangial cells. Increasing concentrations of unlabeled ET-1, BQ123, or S6c were added to mesangial cell membranes and the incubation (at 30° for 60 min) was started by addition of 0.2 nM ^{125}I-ET-1. Bound and free ligands were separated as explained in Experimental Procedures. The 100% binding value represents binding in the absence of any competing ligand and 0% binding represents binding observed in the presence of 1 μM ET-1.

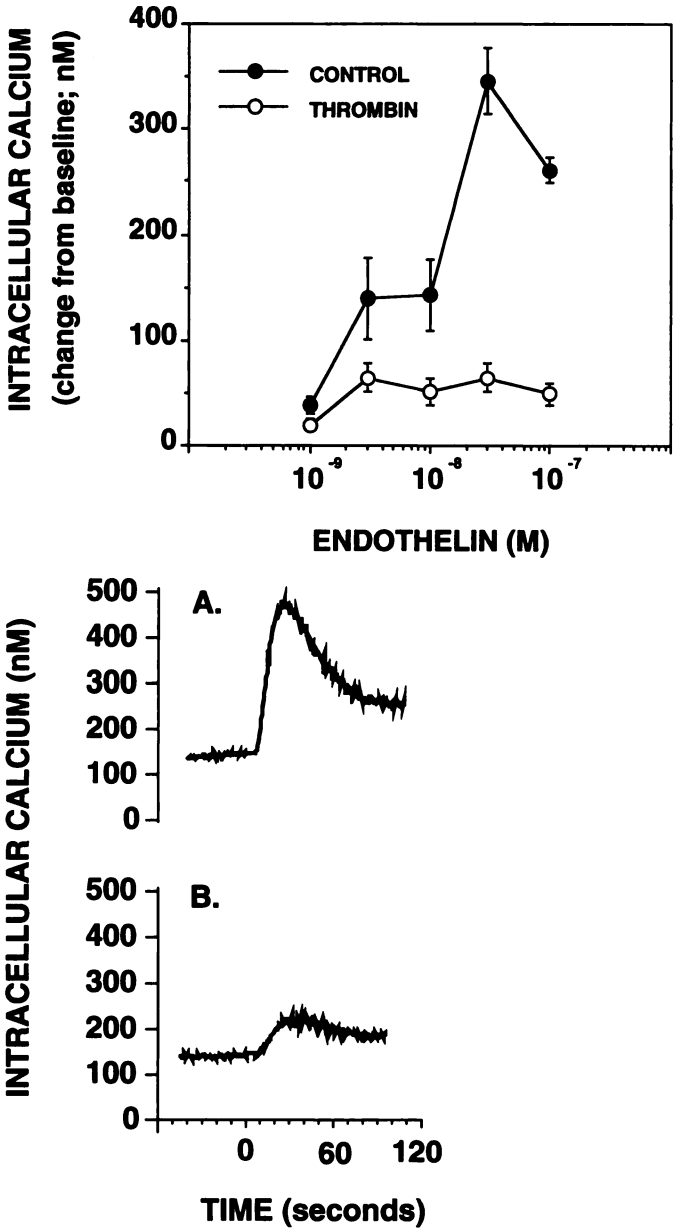


Fig. 4. *Upper*, ET-mediated dose-response curve for intracellular Ca^{2+} mobilization in mesangial cells either without or with a 24-hr pretreatment with 10 nM thrombin. The intracellular Ca^{2+} mobilization was recorded as fluorescent output from fura-2-loaded mesangial cells, as described in Experimental Procedures. The data are presented as a change in $[Ca^{2+}]_i$, compared with base-line (three experiments). *Lower*, representative tracings of intracellular Ca^{2+} mobilization in response to 30 nM ET-1 in mesangial cells either without (A) or with (B) a 24-hr pretreatment with 10 nM thrombin. *Ordinate*, calculated values for $[Ca^{2+}]_i$.

ET-1-stimulated calcium mobilization was measured in thrombin-treated and untreated mesangial cells. ET-1 caused a dose-dependent increase in intracellular Ca^{2+} mobilization in mesangial cells, with an EC_{50} of 11 ± 2 nM (Fig. 4, *upper*). Base-line $[Ca^{2+}]_i$ was 134 ± 4 nM, which was increased 3.5-fold with 30 nM ET-1. Pretreatment of mesangial cells with 10 nM thrombin for 24 hr did not significantly affect base-line $[Ca^{2+}]_i$ (128 ± 4 nM) but decreased the maximum ET-1-stimulated increase in $[Ca^{2+}]_i$ to 65 ± 13 nM above base-line. As indicated in the representative calcium mobilization tracings (Fig. 4, *lower*), the magnitude and the time to peak effects in response to ET-1 were decreased with thrombin pretreatment. To determine whether the thrombin effect was due to a general alteration in the calcium-mobilizing system, the effect of thrombin pretreatment on vasopressin-stimulated calcium mobilization was tested in mesangial cells. Vasopressin (1 μM) stimulated 94 ± 10 and 110 ± 11 nM increases in $[Ca^{2+}]_i$ in untreated and thrombin-treated cells, respectively. The effect of thrombin on ET-stimulated intracellular Ca^{2+} mobilization was, therefore, a result of the specific effect of thrombin on the ET receptor and not a general effect on the calcium-mobilizing systems.

Because the thrombin-mediated decrease in ET receptors might be the result of an increase in the concentration of ET and because it was previously shown that thrombin stimulates the synthesis of ET in mesangial cells (2, 3, 8), experiments were designed to determine whether pretreatment of mesangial cells with ET-1 would result in a similar down-regulation of ET receptors. A 24-hr pretreatment with 100 nM ET-1 resulted in an 85% decrease in ET receptor density, as well as an 84% decrease in ET-1-mediated calcium mobilization. A similar pretreatment with ET-1 did not affect thrombin- or vasopressin-stimulated calcium mobilization (data not shown). In addition, experiments were designed to determine whether thrombin could affect the degradation of ET. NEP activity was measured in untreated mesangial cells and cells treated with thrombin or thrombin plus hirudin. After a 24-hr incubation with 10 nM thrombin, NEP activity decreased from 14.5 ± 0.3 to 10.6 ± 0.2 nmol of product/mg of protein/hr (Fig. 5A). In contrast, there was no difference between control cells and cells pretreated with 30 nM hirudin or hirudin plus 10 nM thrombin (15.9 ± 0.3 and 15.4 ± 0.5 nmol of product/μg of protein/hr, respectively). The effect of thrombin on mesangial cell NEP activity was both time and concentration dependent (Fig. 5, B and C). Treatment with 10 nM thrombin for 16 or 24 hr caused a significant decrease in NEP activity in mesangial cells. Thrombin also caused a dose-dependent decrease in NEP activity, with the maximum effect at 10 nM thrombin. To determine whether this decrease in NEP activity was due to a decrease in NEP protein itself, a Western blot analysis using a specific antibody raised against NEP was performed. The data shown in Fig. 6 indicate that pretreatment of mesangial cells with thrombin resulted in a loss of NEP protein, compared with control cells. Purified NEP was used as the internal standard. To determine whether a decrease in NEP mRNA was the cause of the change in NEP protein, NEP mRNA levels were measured in control mesangial cells and cells treated with thrombin. A Northern blot analysis indicated that thrombin pretreatment caused a decrease in NEP message, compared with control cells (Fig. 7A). Ethidium bromide staining of the gel showed the 28 and 18 S rRNA bands (Fig. 7B). The data were normalized to the amount of rRNA loaded onto the gel by using densitometric scanning and determining a ratio of NEP transcript levels to 28 S rRNA levels. The ratio obtained from control cells was assigned the value of 1. Mesangial cells pretreated with thrombin had a 65–75% decrease in NEP transcripts, compared with control cells (Fig. 7C). To further assess the role of NEP in thrombin-mediated ET receptor down-regulation, experiments were designed to test the effects of thiorphan (a NEP inhibitor) alone and thiorphan in

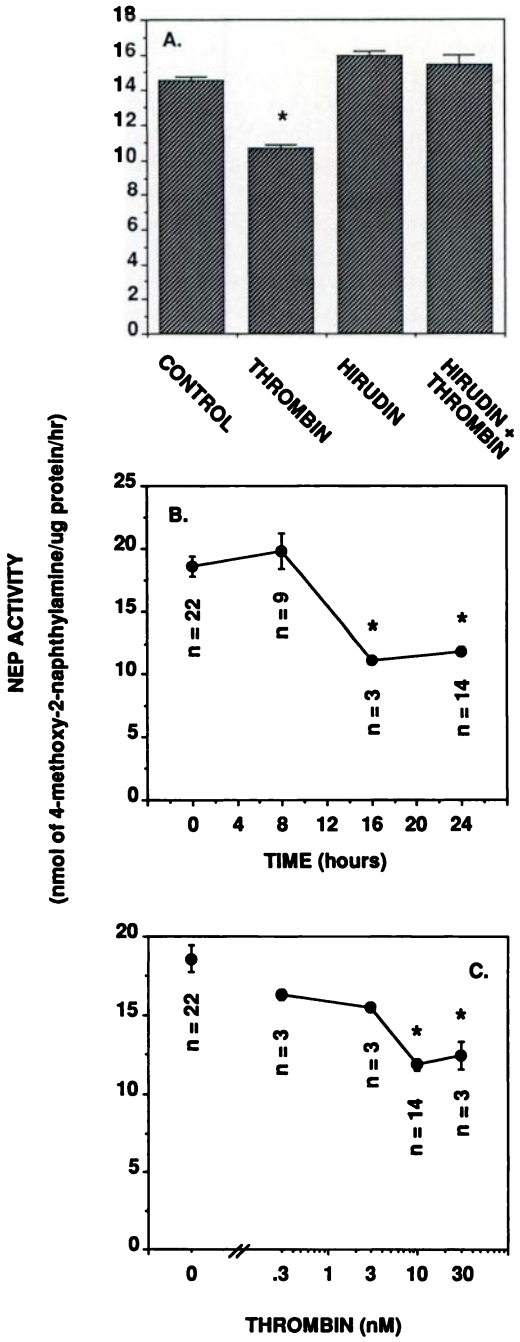


Fig. 5. A, NEP activity in control mesangial cells or in cells after a 24-hr treatment with 10 nM thrombin, 30 nM hirudin, or both (three experiments). B and C, Time-dependent (B) and concentration-dependent (C) effects of thrombin on NEP activity in mesangial cells. $p < 0.05$, compared with control.

combination with thrombin on ET receptor regulation. Pretreatment of mesangial cells with thiorphan alone did not have any effect on ET receptors, whereas thiorphan plus thrombin potentiated the effect of thrombin in down-regulating ET receptors. In the absence of thiorphan, pretreatment of mesangial cells with 1 nM and 3 nM thrombin caused down-regulation of ET receptors by 47% and 57%, respectively. The addition of thiorphan along with 1 and 3 nM thrombin caused a slight potentiation of the ET receptor down-regulation, to 57% and 67%, respectively (data not shown).

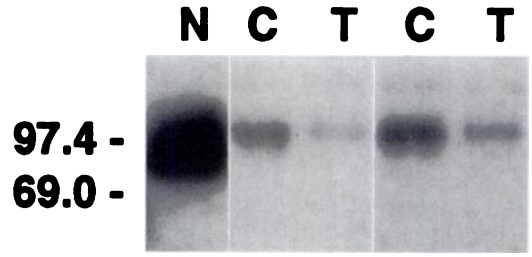


Fig. 6. Western blot analysis of NEP in control mesangial cells (*C*) and in cells treated with 10 nM thrombin for 24 hr (*T*). Purified NEP (*N*) was used as an internal standard. The experiment was performed as described in Experimental Procedures. The data shown are from two experiments with similar results.*

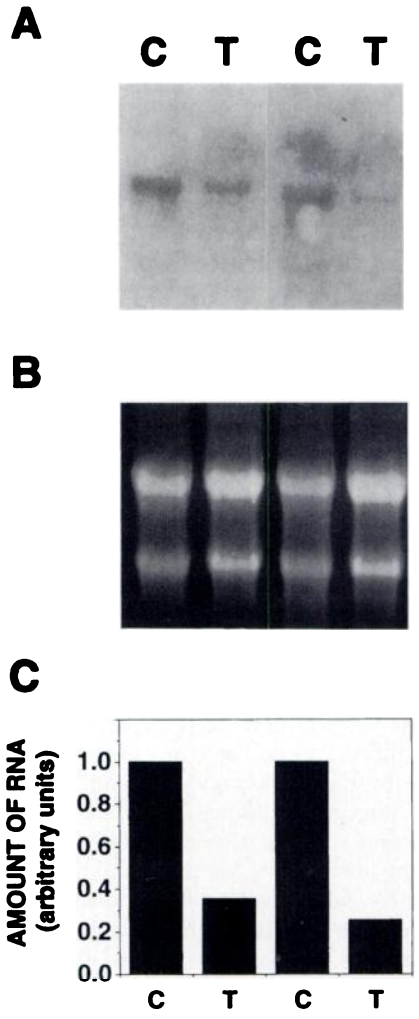


Fig. 7. Northern analysis of mRNA isolated from control mesangial cells (*C*) and cells treated with 10 nM thrombin for 24 hr (*T*). Steady state levels of mRNAs were determined by Northern blotting, as described in Experimental Procedures, using ^{32}P-labeled NEP cDNA probe. A, NEP transcripts from control mesangial cells or cells treated for 24 hr with 10 nM thrombin. B, Ethidium bromide staining of the gel in A, showing the 28 and 18 S rRNAs. C, Results analyzed by densitometry, with the NEP transcripts normalized to the amount of 28 S rRNA. The ratio of NEP to 28 S RNA from control cells was arbitrarily assigned a value of 1.

Discussion

ET is a potent vasoconstrictor that can be synthesized by a variety of cells, including renal cells such as mesangial cells (2, 3, 8), glomerular cells (21), and tubular cells (22). In

mesangial cells, ET stimulates contraction (8–10) and proliferation (6–8), which, *in vitro*, could lead to altered glomerular filtration rates or resistance and glomerular hypertrophy. Indeed, there is growing evidence that ET plays a role in a variety of renal diseases, including ischemia-induced acute renal failure (23), renal ablation (24), cyclosporine nephrotoxicity (25), and radiocontrast nephrotoxicity (26).

Although the precise role of thrombin in the pathogenesis of renal disease is unknown, there is evidence that thrombin is associated with kidney damage. Common features of glomerular nephritides are intraglomerular thrombosis, fibrin deposition, decreased glomerular filtration rate, and glomerular hypertrophy. These features could be associated with the various activities of thrombin. Thrombin is a serine protease in the coagulation cascade and is a key factor in the production of fibrin. In addition to its enzymatic activity, thrombin also acts on specific cellular receptors. In platelets, stimulation of the thrombin receptor results in platelet activation and aggregation, leading to thrombosis. In mesangial cells, thrombin stimulates proliferation (1, 27), which may contribute to glomerular hypertrophy, and also stimulates contraction (1), which could decrease the filtration surface of the glomerulus. All of these effects could result in altered renal function.

The present study demonstrates that thrombin caused a decrease in ET receptors in mesangial cells, which display predominantly ET_A receptors. The effect, which was due to a decrease in receptor number without a change in affinity, was abolished by co-treatment with hirudin, a specific inhibitor of thrombin activity. Functionally, the thrombin-mediated decrease in receptor number resulted in a suppression of ET-stimulated intracellular Ca^{2+} mobilization. This functional effect was specific for ET, because thrombin pretreatment did not affect vasopressin-stimulated intracellular Ca^{2+} mobilization in mesangial cells. The apparent K_d values of ^{125}I-ET-1 for the ET receptors were 89 and 67 pM in membranes prepared from control and thrombin-treated mesangial cells, respectively, whereas the EC_{50} value for the stimulation of intracellular Ca^{2+} release was 10 nM. This large discrepancy between the two values may be due to the fact that the binding assays were done with membranes, whereas the functional studies were done with intact cells. When saturation binding experiments were performed in intact cells, the observed K_d was 0.4–0.6 nM. This difference in K_d values for whole-cell and membrane binding is not surprising, because in intact cells the effect of intracellular GTP might decrease the affinity of the ligand, whereas in membrane binding assays there is no contribution by GTP. Comparison of a K_d value of 0.5 nM with an EC_{50} value of 11 nM indicates a 20-fold difference between binding and function, which is not unusual.

The thrombin-mediated down-regulation of ET receptors in mesangial cells probably results from an increase in ET levels stimulated by thrombin. In fact, human mesangial cells have been shown to constitutively express the ET gene (2), and thrombin, transforming growth factor-β, and a thromboxane analog (U-46619) stimulate an increase in human mesangial ET mRNA and immunoreactive ET. Rat mesangial cells also respond to thrombin with an increase in immunoreactive ET-1 (3, 4). However, thrombin-mediated down-regulation of ET binding and function may be due not only to thrombin-stimulated ET synthesis but also to a decrease in ET degradation. As explained in Results, the contribution of NEP to thrombin-mediated down-regulation of ET receptors was assessed by pretreating mesangial cells with NEP inhibitor alone, as well as by adding NEP inhibitor along with thrombin. Although NEP inhibitor alone did not cause ET receptor down-regulation, it potentiated thrombin-mediated down-regulation of ET receptors. The lack of an effect of NEP inhibitor alone on ET receptors is not surprising, because mesangial cells produce very little ET-1 under unstimulated conditions and inhibition of NEP under these conditions is therefore ineffective in down-regulating ET receptors.

NEP (24.11) is an ectoenzyme present at high levels in the kidney (28) that degrades peptide hormones such as ET, atrial natriuretic factor, and enkephalins. The results of the present study show that thrombin decreases NEP mRNA, protein, and activity. This effect may contribute to a thrombin-mediated increase in ET levels, which in turn causes a decrease in ET receptors. The ability of thrombin to both enhance ET synthesis and decrease ET degradation suggests that thrombin may play a major role in the physiological regulation of ET in mesangial cells.

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