5-HT2A Receptor Induces ERK Phosphorylation and Proliferation through ADAM-17 Tumor Necrosis Factor-α-converting Enzyme (TACE) Activation and Heparin-bound Epidermal Growth Factor-like Growth Factor (HB-EGF) Shedding in Mesangial Cells*

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In this study, we present multiple lines of evidence to support a critical role for heparin-bound EGF (epidermal growth factor)-like growth factor (HB-EGF) and tumor necrosis factor-α-converting enzyme (TACE) (ADAM17) in the transactivation of EGF receptor (EGFR), ERK phosphorylation, and cellular proliferation induced by the 5-HT2A receptor in renal mesangial cells. 5-hydroxy-tryptamine (5-HT) resulted in rapid activation of TACE, HB-EGF shedding, EGFR activation, ERK phosphorylation, and longer term increases in DNA content in mesangial cells. ERK phosphorylation was attenuated by 1) neutralizing EGFR antibodies and the EGFR kinase inhibitor, AG1478, 2) neutralizing HB-EGF, but not amphiregulin, antibodies, heparin, or CM197, and 3) pharmacological inhibitors of matrix-degrading metalloproteinases or TACE small interfering RNA. Exogenously administered HB-EGF stimulated ERK phosphorylation. Additionally, TACE was co-immunoprecipitated with HB-EGF. Small interfering RNA against TACE also blocked 5-HT-induced increases in ERK phosphorylation, HB-EGF shedding, and DNA content. In aggregate, this work supports a pathway map that can be depicted as follows: 5-HT → 5-HT2A receptor → TACE → HB-EGF shedding → EGFR → ERK → increased DNA content. To our knowledge, this is the first time this pathway has been implicated in 5-HT-induced EGFR transactivation or in proliferation induced by a G protein–coupled receptor in native cells in culture.

Cells receive environmental cues through various cell surface receptors, which allow extracellular stimuli to regulate intracellular signaling pathways. Two ubiquitous classes of cell surface receptors are those that couple to G proteins (GPCRs)2 and those that possess intrinsic receptor tyrosine kinase (RTK) activity. Despite the importance of these two classes of receptors, our knowledge of how they coordinate cellular signals is incomplete. Previously, signals generated by RTKs and GPCRs were thought to be cleanly compartmentalized, with little or no cross-talk between the signaling pathways. However, recently, the lines of distinction between signaling pathways used by GPCRs and RTKs have become less definite. There is a new awareness that RTKs such as the epidermal growth factor (EGF) receptor (EGFR) and GPCRs possess significant potential for cross-talk during signal initiation and propagation. Cross-talk can take the form of shared signaling pathways (1), or for GPCRs, using the RTKs as signaling platforms (2–4). Thus, contrary to relatively recent dogma, it is now quite clear that RTKs and GPCRs engage in extensive cross-talk with each other.

Receptor transactivation or inter-receptor cross-talk can involve complex mechanisms such as in the case of GPCR-induced EGFR transactivation. In that regard, some aspects of the molecular mechanisms of GPCR-induced transactivation of RTKs have recently been elucidated. Current studies have highlighted the potential importance of matrix metalloproteinases (MMPs) and members of the ADAM (cell surface enzymes that contain a disintegrin and metalloprotease domain) family, especially ADAM-9, -10, -12, and -17, in GPCR-induced transactivation of the EGFR (5). Recent work supports the concept that ADAMs and MMPs process cell surface–bound growth factors such as heparin-bound EGF-like growth factor (HB-EGF), amphiregulin, or TGFα, releasing them from cell surface “tethers,” thus providing ligands for the EGFR (6, 7). Many details of GPCR-induced activation of these enzymes and the

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2 The abbreviations used are: GPCR, G protein-coupled receptor; EGF, epidermal growth factor; EGFR, EGF receptor; HB-EGF, heparin-bound EGF-like growth factor; 5-HT, 5-hydroxy-tryptamine; TGF, transforming growth factor; TACE, tumor necrosis factor α-converting enzyme; TACEsi, TACE-silenced cells; CsI, control-silenced cells; siRNA, small interfering RNA; ERK, extracellular signal-regulated kinase; RTK, receptor tyrosine kinase; MMP, matrix metalloproteinase; PMA, phorbol 12-myristate 13-acetate; HBSS, Hanks’ balanced salt solution; HSPG, heparan sulfate proteoglycan.
release of their substrates remain undefined, at least partially because there are cell type- and GPCR-specific variables of the transactivation process (8, 9).

ADAMs comprise a relatively new family of membrane-bound cell surface proteins that participate in diverse functions such as fertilization, development, and cellular proteolytic activities such as ectodomain shedding (10, 11). Like many other proteins, ADAMs are composed of distinct modules (7, 12). ADAMs have an amino-terminal “pro” module that maintains the adjacent protease-like module in an inactive conformation. The pro domain can be cleaved from the protease module via the actions of furin. The ADAM proteins also contain (moving from amino to carboxyl terminus) a disintegrin module, a cysteine-rich region, EGF repeats, a transmembrane region, and a cytoplasmic domain (7, 12). There are currently at least 23 members of this family of proteins, but the potential candidates for the pro-HB-EGF protease can be narrowed down relatively easily. For example, only about half (ADAM-1, -8, -9, -10, -12, -13, -15, -16, -17, -19, and -20) actually contain a functional proteolytic site (HEXGHXGXXHD) (10). Of those, many have very restricted tissue distributions or have been identified only in non-mammalian species. Only a few ADAMs (1, 9, 15, and 17) are broadly distributed (10). To this point, there have been few organized attempts to dissect the role of specific ADAMs in the cross-talk between GPCRs and EGFR in native tissues or non-transformed cells. In that regard, one group of investigators showed that different ADAM enzymes and various EGF-like growth factors can contribute to transactivation depending upon the cell system used. For example, lysophosphatidic acid induces EGFR activation through ADAM-15 and amphiregulin or TGFα (13), whereas ADAM-17 and HB-EGF appear to be necessary for the cross-talk between the angiotensin-II AT1 receptor and EGFR in kidney tumor cells and in Cos7 cells (9).

Because most of the previous studies were carried out in transformed cell lines, our aim in the present study was to investigate the cross-talk between an endogenous GPCR, namely the 5-HT₂A receptor, and EGFR, with particular emphasis on the involvement of endogenous ADAM enzymes and EGF-like growth factors in a more physiological environment using mesangial cells as our model system. Our laboratory previously showed that the 5-HT₂A receptor transactivates EGFR via protein kinase C in mesangial cells, although the specific pathway of transactivation was not characterized (14). Glomerular mesangial cells, which have a combined smooth muscle and fibroblast-like phenotype, play important roles under physiological and pathophysiological conditions in regulating glomerular extracellular matrix. Because mesangial cells reside in a confined environment, regulation of proliferation and extracellular matrix by growth factors is tightly regulated. Renal mesangial cells express many mitogenic GPCRs, including angiotensin II (15), bradykinin (15), lysophosphatidic acid (16), and 5-HT₂A receptors (17). Mesangial cells also express RTKs, which may participate in the proliferative phase of chronic renal failure (18) or in the recovery from renal failure. Mesangial cells possess an EGF receptor (19) and also express HB-EGF (20). Thus, we chose this cell model for our studies.

EXPERIMENTAL PROCEDURES

Chemicals and Antibodies—All cell culture media, fetal bovine serum and antibiotics, 4−12% acrylamide gels, SDS sample buffer, and sample reducing agent were purchased from Invitrogen. Insulin, bovine serum albumin, 5-HT, HB-EGF, and EGF were from Sigma; GM6001, MMP inhibitor II, MMP inhibitor 9/13, AG1478, protease inhibitor mixture set III, and the diphtheria toxin analogue CM197 were acquired from Calbiochem. Neutralizing antibodies against HB-EGF, TGFα, betacellulin, and amphiregulin and fluorogenic peptide substrates were purchased from R&D Systems; EGFR antibodies were from Upstate and Cell Signaling, and rat EGFR neutralizing antibody was from the Developmental Studies Hybridoma Bank, The University of Iowa. TACE antibodies were purchased from Chemicon (AB19027) and R&D Systems (AB930). ADAM9 antibody was from Zymed Laboratories Inc., ADAM10 antibody was from Chemicon, and ERK and phospho-ERK antibodies were from Cell Signaling. TACE blocking peptide was from Chemicon. Protein A/G beads were purchased from Santa Cruz Biotechnology. Chemiluminescence reagent was from Pierce. The pLentiLox 3.7 vector was kindly provided by Dr. Luk van Parijs (Massachusetts Institute of Technology).

Cell Culture—Primary rat mesangial cells were obtained from cortical sections of kidneys from 100−150-g Sprague-Dawley rats by collagenase treatment and a standard sieving technique as described previously (21). The kidneys were harvested in accordance with protocols approved by the Institutional Animal Care and Use Committees of the Medical University of South Carolina and the Ralph H. Johnson Veterans Affairs Medical Center. Cells were cultured in RPMI1640 medium supplemented with 20% fetal bovine serum and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Cells were subcultured weekly and used between passages 6 and 14. Human mesangial cells were a gift from Dr. Hanna Abboud (University of Texas Health Science Center at San Antonio). The cells were maintained in medium containing a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F12 supplemented with 17% fetal bovine serum, 26 μg/ml insulin, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37 °C in a humidified 5% CO₂ atmosphere. The cells were subcultured weekly and used between passage numbers 9−20.

RNA Interference and Lentiviral Infection—We used the pLentiLox 3.7 vector (LL3.7), which contains the mouse U6 promoter, which is an RNA polymerase III promoter that efficiently directs the transcription of short RNAs (22). This vector also expresses green fluorescent protein from a cytomegalovirus promoter, which allows for monitoring of the efficiency of infection. Because the TACE siRNA sequences used previously by others are not suitable for expression in viral systems, we designed and tested alternate siRNA sequences. Small hairpin RNAs were designed to contain 19−23-nucleotide sense sequences identical to the target molecule(s), followed by a short (9-nucleotide) nonspecific loop sequence and an antisense sequence, followed by five thymidines, which serve as a stop signal for RNA polymerase III. The 5'-phosphorylated, PAGE-
purified oligonucleotides (Sigma Genosys) were annealed and cloned into pHL3.7 between the HpaI and XhoI sites. The vector was transfected into 293FT cells (Invitrogen) together with the viral packaging vectors by lipid transfection (Transfectin, Bio-Rad), and the 48-h supernatant of the cells was used for human mesangial cell infection. One small hairpin RNA construct that resulted in nearly complete inhibition of TACE expression in human mesangial cells was chosen for use in our studies. This construct targets the sequence 5′-ggatcttggcaagtgtaag-3′ (positions 1867–1885) in ADAM-17 splice variant 2. As a control, we used 19-nucleotide scrambled small hairpin RNA, which did not give more than a 16-nucleotide match against any human genomic sequence.

Cell Signaling Experiments—For ERK phosphorylation studies, human and rat mesangial cells were seeded into 12-well culture plates (Falcon) and serum-starved for 2 days in 0.5% bovine serum albumin-containing medium. For neutralizing EGFR activation, cells were preincubated with 25 μg/ml EGFR antibody for 1 h. For MMP and EGFR kinase inhibition studies, cells were pretreated for 30 min with the indicated inhibitor (10 μM GM6001; 5 μM MMP inhibitor II and MMP inhibitor 9/13 or 100 nM AG1478) for 5 or 10 min prior to treatment with the agonists (1 μM 5-HT, 1–10 ng/ml HB-EGF, and 1–10 ng/ml EGF). Neutralizing antibodies (10 μg/ml against HB-EGF, TGFα, betacellulin, and amphiregulin) and the diphtheria toxin analogue CM197 (100–200 ng/ml) were applied 30 min prior to agonist treatment.

Immunoprecipitation—Immunoprecipitations from human and rat mesangial cells were performed as follows. The cells were grown to confluency in 10-cm dishes and lysed in 1 ml/dish of radiolmmune precipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% Nonidet P-40, 1 mM EDTA) supplemented with protease inhibitors (1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 0.8 μM aprotinin, 50 μM bestatin, 15 μM E-64, 20 μM leupeptin, 10 μM pepstatin A, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and 1 mM phenylmethylsulfonyl fluoride). Equal amounts of protein (1 mg) were precleared with protein A/G-Sepharose beads at 4°C. After a brief centrifugation, the supernatants were removed and incubated with 6 μg of HB-EGF antibody (Oncogene), 8 μl of TACE antiserum (R&D Systems), or 6 μg of EGFR antibody (Upstate Biotechnology) overnight at 4°C, respectively. The next day, 50 μl of protein A/G beads was added, and the samples were further incubated at 4°C for 1 h. Then, the samples were centrifuged and washed three times with 1 ml of radiolmmune precipitation buffer, and the proteins were eluted from the beads using 2× Laemmli sample buffer.

Western Blot Analysis—Protein samples were boiled for 3 min in reducing SDS-sample buffer and separated by 4–12% or by 6% acrylamide gels. Resolved proteins were transferred to 0.45-μm polyvinylidene difluoride membranes (Millipore), blocked with 4% nonfat dry milk or 3% bovine serum albumin for 1 h, and incubated overnight at 4°C with the following antibodies: 1:1000 ERK and phospho-ERK (Cell Signaling), 1 μg/ml HB-EGF (Oncogen), 1:500 ADAM9 (Zymed Laboratories Inc.) and ADAM10 (Chemicon), 1:1000 EGFR (Cell Signaling), 1:3000 α-actinin (Santa Cruz Biotechnology), or 1:2000 TACE (R&D Systems) antibodies. Membranes were washed three times in phosphate-buffered saline containing 0.1% Tween 20 and incubated for 1 h at room temperature in appropriate dilutions of secondary antibodies. Immunoreactive protein bands were visualized using enhanced chemiluminescence and recorded on Kodak BioMax XR film. Optimally exposed autoradiographs were digitally scanned and analyzed using NIH Image software.

Fluorescence Enzymatic Activity Measurement—Control and TACE-silenced human mesangial cells were seeded into 96-well plates and incubated overnight. Growth medium was removed the next day and replaced with Hanks’ balanced salt solution (HBSS) containing 0, 1, and 10 μM 5-HT or 1 μM PMA (Sigma) and 6 μM fluoroogenic peptide substrate (R&D Systems). Enzymatic activities were measured after 10 min of incubation at room temperature using a Molecular Devices SpectraMax instrument and SoftMaxPro software. As negative control, HBSS alone, fluoroergic peptide substrate in HBSS, or fluoroergic peptide substrate and 1 or 10 μM 5-HT or 1 μM PMA without cells were used.

Cell Proliferation Assay—Control- and TACE-silenced human mesangial cells (5000/well) were seeded into 96-well plates. The cells were starved in 0.1% serum-containing Dulbecco’s modified Eagle’s medium/F12 medium for 24 h. Then, the cells were treated in 1% serum-containing Dulbecco’s modified Eagle’s medium/F12 with vehicle or 1 and 10 μM 5-HT or kept in regular growth medium. The media were changed every second day. After 6 days, the media were removed, and the cells were placed to 80°C. The DNA content of the wells was measured using FluoReporter blue (Molecular Probes) cell proliferation kit as suggested by the manufacturer (23). The cells were freeze-thawed, following which aqueous Hoechst dye was added, and fluorescence (360/460 nm) was measured.

Statistical Analysis—Student’s t test and analysis of variance using GraphPad statistics software were performed to determine statistical significance. p values < 0.05 were regarded as statistically significant.

RESULTS

Activation of the G protein-coupled 5-HT<sub>2A</sub> Receptor Induces ERK Phosphorylation through the EGFR—We tested the involvement of EGFR transactivation in the activation of the mitogen-activated protein kinase, ERK, by the serotonin 5-HT<sub>2A</sub> receptor in rat renal mesangial cells. Fig. 1A shows that when rat renal mesangial cells were pretreated with a neutralizing antibody against the EGFR (25 μg/ml) for 1 h followed by treatment for 10 min with 1 μM 5-HT, there was nearly complete attenuation of 5-HT-induced ERK phosphorylation (shown in the figure legends as p-ERK). As a control, we treated cells with 10 ng/ml EGF and showed that the neutralizing EGFR antibody also inhibited EGF-mediated ERK phosphorylation, although to a lesser degree (~37%). 5-HT treatment detectably increased ERK tyrosine phosphorylation in EGFR immunoprecipitates, albeit to a smaller extent than a saturating concentration of exogenously applied EGF. This phenomenon has been observed numerous times with many different GPCRs (24). We hypothesize that the difference reflects the fact that endogenous EGF-like growth factor (for example, HB-EGF) release in response to 5-HT is limiting and produces submaxi-
normal EGFR activation when compared with a maximally efficacious concentration of EGF.

To further demonstrate that phosphorylation of the EGFR is required for 5-HT-induced ERK activation, we pretreated mesangial cells with AG1478, a compound that inhibits EGFR kinase activity and autophosphorylation. As seen in Fig. 1B, AG1478 pretreatment markedly reduced the 5-HT-induced ERK phosphorylation when compared with vehicle (Me2SO) control. EGF-induced phosphorylation of ERK was completely attenuated by 100 nM AG1478. The corresponding changes in EGFR phosphorylation are depicted in Fig. 1B, bottom. Thus, two lines of evidence (antibody neutralization and pharmacological inhibition) implicate the EGFR in 5-HT2A receptor-induced ERK phosphorylation in rat mesangial cells.

Involvement of HB-EGF in 5-HT2A and EGFR Cross-talk—To test whether EGF-like growth factors are involved as extracellular soluble factors in the transactivation of EGFRs, we first employed heparin, a glycosaminoglycan whose structure is similar to the heparan sulfate proteoglycan (HSPG) molecules present on the cell surface. HSPG serves as co-receptor for many growth factor receptors (25–27). Heparin can act as a competitor for HSPG and displace HB-EGF or other HSPG-tethered molecules such as amphiregulin (28). As shown in Fig. 2A, 100 µg/ml heparin completely inhibited 5-HT2A receptor-induced ERK phosphorylation in rat mesangial cells. It had no effect on EGF-induced ERK activation (data not shown).

Pro-HB-EGF is the unprocessed form of the HB-EGF growth factor and is the only known receptor for diphtheria toxin (29, 30). To test specifically whether HB-EGF is involved in ERK phosphorylation induced by 5-HT, we employed CM197, a non-toxic analogue of diphtheria toxin. This compound has been shown to inhibit tyrosine phosphorylation of EGFR by a variety of GPCR ligands (4). We pretreated human mesangial cells with 100 or 200 ng/ml CM197 for 1 h and then stimulated them with 1 µM 5-HT. At both concentrations, the toxin significantly (40 and 45%) attenuated 5-HT-mediated ERK phosphorylation, suggesting that HB-EGF is involved in the transactivation of EGFR by the 5-HT2A receptor in mesangial cells (Fig. 2B).

To confirm the role of HB-EGF, we employed neutralizing antibody against the two most widely studied EGF family growth factors, HB-EGF and amphiregulin (Fig. 2C). Pretreatment with a neutralizing HB-EGF antibody showed that ERK phosphorylation by 5-HT was reduced by about 50%. In contrast, there was no attenuation of the signal when we employed a neutralizing amphiregulin antibody. Exposure to exogenous HB-EGF at concentrations of 1 and 10 ng/ml induced concentration-dependent increases in ERK phosphorylation. This effect was partially (45%) antagonized by the neutralizing HB-EGF antibody (10 ng/ml), consistent with the results for the 5-HT2A receptor (data not shown).

MMP/ADAM Activity Is Necessary for 5-HT-induced ERK Activation—To investigate the involvement of MMPs and/or ADAMs in ERK phosphorylation by 5-HT, we employed the broad spectrum metalloenzyme inhibitor GM6001. As a control, we used a similar compound that does not have MMP inhibitory properties (GM6001 control). As shown in Fig. 3A, 10 µM GM6001 significantly reduced 1 µM 5-HT-induced ERK phosphorylation with no significant effect on EGF-induced ERK activation. GM6001 also reduced ERK phosphorylation in unstimulated cells, suggesting that constitutive MMP activity maintains basal ERK activation in mesangial cells (see Fig. 7). The effects of two additional MMP inhibitors are depicted in Fig. 3B. As shown, treatment with 5 µM MMP inhibitor II (which inhibits MMPs 1,3,7, and 9) or MMP inhibitor 9/13 (which inhibits MMPs 1,3,7,9, and 13) both suppressed 5-HT-

FIGURE 1. Activation of the G protein-coupled 5-HT2A receptor induces ERK phosphorylation through the EGFR. A, rat mesangial cells were preincubated with either control IgG (C IgG) or neutralizing EGFR antibody (nEGFR Ab, 25 µg/ml) and stimulated for 10 min with 1 µM 5-HT or 10 ng/ml EGF. Cell lysates were immunoblotted against phosphorylated ERK (p-ERK) and total ERK. EGFR was immunoprecipitated from the lysate of untreated and treated cells and blotted for phosphotyrosine (P-Tyr) and total EGFR. Results are mean ± S.E. (n = 3), *, p < 0.05; **, p < 0.01 versus 5-HT IgG control; #, p < 0.05; ##, p < 0.01 versus EGF IgG control. B, rat mesangial cells were preincubated with 100 nM AG1478 or Me2SO (DMSO) (control) and stimulated for 10 min with 1 µM 5-HT or 10 ng/ml EGF. Cell lysates were immunoblotted against phosphorylated ERK and total ERK. Again, as a control, EGFR was immunoprecipitated from untreated and treated cell lysates and blotted for phosphotyrosine and total EGFR. Results are mean ± S.E. (n = 3), *, p < 0.05; **, p < 0.01 versus 5-HT Me2SO control; #, p < 0.05; ##, p < 0.01 versus EGF Me2SO control.
induced ERK activation without affecting EGF-induced activation. Although these data suggest that the non-ADAM MMPs 1,3,7, or 9 might mediate cross-talk between the 5-HT2A receptor and the EGFR, their specificity varies depending on drug concentration, treatment time, and cell types, and their activity against ADAMs has not been specifically determined. Moreover, the only non-ADAM MMP that has been implicated in EGFR transactivation is MMP7 (31), which is not expressed in human mesangial cells (data not shown). Interestingly, the molecular size(s) of the strongest TACE immunoreactive bands is/are higher (~250 kDa) than the predicted core molecular size (80–130 kDa), of which we had only traces present (Fig. 4A). To verify that the band(s) represented authentic TACE, we preincubated the TACE antibody (R&D Systems) with a blocking peptide prior to immunoprecipitation from human mesangial cell lysates. Fig. 4B shows that the peptide significantly attenuated the intensity of TACE immunopositive bands on a TACE Western blot (R&D Systems) when compared with the precipitate of non-treated antibody. The intensity of the bands (250, 150, 130, and 85 kDa) decreased by 58, 74, 87, and 65%, respectively. In another experiment, we used control IgG or TACE antibody to immunoprecipitate ADAM17 (R&D Systems) and then probed with a different anti-TACE antibody (Chemicon). The result is depicted in Fig. 4C, which shows a similar immunoreactive pattern to that shown in Fig. 4A, supporting the idea that the band(s) represent TACE. Furthermore, control IgG used in the immunoprecipitation step did not yield any immunoreactive bands. These data taken together strongly support that the 250-kDa band(s) on Fig. 4A indeed represent(s) TACE. Based on these results, we believed that TACE was a reasonable candidate to be the membrane-bound sheddase that is activated following 5-HT treatment.

**FIGURE 2.** Involvement of HB-EGF in 5-HT2A and EGFR cross-talk. A, rat mesangial cells were stimulated with 1 μM 5-HT with or without heparin (100 μg/ml) for 10 min. Cell lysates were immunoblotted against phosphorylated ERK (p-ERK) and total ERK. Results are presented as mean ± S.E. (n = 3), **p < 0.01 versus 5-HT alone.** C, control. B, human mesangial cells were preincubated with various concentrations of the diphertheria toxin analogue CM197 (100 or 200 ng/ml) for 30 min and stimulated with 1 μM 5-HT for 10 min. Cell lysates were immunoblotted against phosphorylated ERK and total ERK. Results are presented as mean ± S.E. (n = 3), *p < 0.05 versus 5-HT alone. C, rat mesangial cells were preincubated with neutralizing HB-EGF antibody (nHB-EGF Ab, 50 ng/ml) or with neutralizing amphiregulin antibody (nAR Ab, 50 ng/ml) or with the same concentration of control IgG (C IgG). Cell lysates were immunoblotted against phosphorylated ERK and total ERK. Results are presented as mean ± S.E. (n = 3), *p < 0.05 versus 5-HT C IgG alone.

**FIGURE 3.** MMP activity is necessary for 5-HT-induced ERK phosphorylation. The involvement of metalloproteinases is shown by the effects of GM6001 and its inactive control compound (GM control) (A) or MMP inhibitor II and MMP inhibitor 9/13 (B). Rat mesangial cells were preincubated with either MMP inhibitors (MMP Inhib II and MMP Inhib 9/13) or Me2SO (DMSO) control for 30 min and treated with 1 μM 5-HT or 10 ng/ml EGF. Cell lysates were immunoblotted against phosphorylated ERK (p-ERK) and total ERK (ERK). Results are mean ± S.E. (n = 3), **p < 0.05 versus 5-HT Me2SO and 5-HT GM control; #, p < 0.05 versus Me2SO and GM control.

Co-immunoprecipitation of HB-EGF with Matrix Degrading Metalloproteinases—We first tested whether ADAM family MMPs would co-immunoprecipitate with HB-EGF from mesangial cell lysates. Immunoprecipitates were resolved by SDS-PAGE and probed with antibodies against ADAMs that have been implicated in GPCR-RTK cross-talk: ADAMs 9, 10, 12, and 17 (32–35). Only TACE (ADAM17) immunoreactivity was enriched in HB-EGF precipitates from human (H) and rat (R) mesangial cells (Fig. 4A). We were unable to significantly enrich any of the other ADAMs that we investigated (data not shown). We performed a reverse immunoprecipitation, which showed that HB-EGF co-immunoprecipitated with TACE (data not shown). Interestingly, the molecular size(s) of the strongest TACE immunoreactive bands is/are higher (~250 kDa) than the predicted core molecular size (80–130 kDa), of which we had only traces present (Fig. 4A). To verify that the band(s) represented authentic TACE, we preincubated the TACE antibody (R&D Systems) with a blocking peptide prior to immunoprecipitation from human mesangial cell lysates. Fig. 4B shows that the peptide significantly attenuated the intensity of TACE immunopositive bands on a TACE Western blot (R&D Systems) when compared with the precipitate of non-treated antibody. The intensity of the bands (250, 150, 130, and 85 kDa) decreased by 58, 74, 87, and 65%, respectively. In another experiment, we used control IgG or TACE antibody to immunoprecipitate ADAM17 (R&D Systems) and then probed with a different anti-TACE antibody (Chemicon). The result is depicted in Fig. 4C, which shows a similar immunoreactive pattern to that shown in Fig. 4A, supporting the idea that the band(s) represent TACE. Furthermore, control IgG used in the immunoprecipitation step did not yield any immunoreactive bands. These data taken together strongly support that the 250-kDa band(s) on Fig. 4A indeed represent(s) TACE. Based on these results, we believed that TACE was a reasonable candidate to be the membrane-bound sheddase that is activated following 5-HT treatment.
Fluorogenic TACE Substrate Cleavage Is Increased by 5-HT Treatment—To study changes in the metalloproteinase activity induced by 5-HT in mesangial cells, we used a quenched fluorogenic peptide substrate that is known to be cleaved by TACE. We observed in real time a ~100% increase in enzymatic activity in human mesangial cells treated with 1 μM 5-HT when compared with untreated cells (Fig. 5). In contrast, there was no increase in fluorescence when we used “CatE1,” which is an ADAM substrate that is not cleaved by ADAM-17 (Fig. 5). As a positive control, we employed the phorbol ester PMA, which is known to non-selectively increase MMP activity. PMA induced more than a 3-fold increase in TACE substrate fluorescence but had no effect on CatE1 fluorescence.

Silencing TACE Expression Inhibits 5-HT-induced ERK Phosphorylation—To further support a role for TACE in 5-HT-induced ERK phosphorylation, we introduced silencing RNAs targeting TACE into human mesangial cells by the means of a lentiviral vector. Successful silencing was confirmed by Western blotting 4 days after infection. As depicted in Fig. 6A, expression of both the 130-kDa and the 85-kDa forms of the enzyme were almost completely inhibited, and the expression of the 250-kDa band was significantly attenuated (~50%) in TACE-silenced cells (TACEsi) when compared with control-silenced cells (Csi). There was no change in the expression of the 150-kDa band, which may represent a TACE splice variant not recognized by our siRNA. Actinin expression was used as a loading control. ADAMs 9, 10, and 12 expression was unaffected in cells infected with Csi and TACEsi, confirming that TACE silencing had no effect on the synthesis of these proteins (data not shown). To determine whether TACE silencing affected 5-HT_{2A} receptor signaling, control- and TACE-silenced human mesangial cells were stimulated with 1 μM 5-HT for 5 min, and ERK phosphorylation was assessed by Western blotting (Fig. 6B). The 5-HT-induced ERK phosphorylation was completely attenuated in TACE-silenced cells when compared with controls. On the other hand, TACE silencing had no effect on the 1 ng/ml EGF-induced ERK activation.

TACE Silencing Attenuates 5-HT-induced HB-EGF Shedding—During GPCR stimulation, cellular pro-HB-EGF, which is tethered to the plasma membrane by its transmembrane domain, is processed by a metalloenzyme to mature HB-EGF (4). Since we
hypothesized that TACE is the metalloenzyme that processes HB-EGF in response to 5-HT$_{2A}$ receptor stimulation, we investigated the role of TACE in HB-EGF shedding of human mesangial cells. Control- and TACE-silenced cells were stimulated with 1 $\mu$M 5-HT for 1 h, and the released HB-EGF was evaluated from the cell medium by Western blotting. Results are mean $\pm$ S.E. (n = 3), **, p < 0.01 versus 5-HT, 1B, immunoblot.

FIGURE 7. TACE silencing attenuates 5-HT-induced HB-EGF shedding. Csi and TACEsi human mesangial cells were treated with 1 $\mu$M 5-HT for 1 h, and the released HB-EGF was evaluated from the cell medium by Western blotting. Results are mean $\pm$ S.E. (n = 3). **, p < 0.01 versus Csi 5-HT.

DISCUSSION

We present multiple lines of evidence that support a central role for TACE (ADAM17) and HB-EGF in the transactivation of EGFR receptors by the 5-HT$_{2A}$ receptor in renal mesangial cells and in cellular proliferation. Stimulation of the 5-HT$_{2A}$ receptor leads to rapid activation of TACE, shedding of HB-EGF, and activation of EGFRs. Transactivation of the EGFRs results in ERK phosphorylation and proliferation of mesangial cells. To our knowledge, this is the first time that TACE has been implicated in EGFR transactivation in native cells in culture or in proliferation induced by a 5-HT. The molecular mechanisms underlying cross-talk between GPCRs and RTKs are being intensively studied. We recently showed that 5-HT$_{2A}$ receptors and other GPCRs rapidly activated EGFRs in mesangial cells, following which the EGFR desensitized (36). The pathway(s) through which those events occur heretofore have not been described. The current study addresses that gap in our knowledge base.

The pathway controlling serotonin-stimulated mesangial cell proliferation can be depicted as follows: 5-HT $\rightarrow$ 5-HT$_{2A}$ receptor $\rightarrow$ TACE $\rightarrow$ HB-EGF $\rightarrow$ EGFR $\rightarrow$ ERK $\rightarrow$ proliferation. We used a neutralizing antibody and the EGFR kinase inhibitor, AG1478, to support a role for the EGFR in 5-HT$_{2A}$-mediated ERK activation. A role for HB-EGF was supported by: 1) attenuation of 5-HT$_{2A}$-induced ERK phosphorylation by neutralizing HB-EGF antibodies, 2) attenuation of ERK phosphorylation by heparin, 3) attenuation of ERK phosphorylation by CM197, 4) induction of ERK phosphorylation by exogenously administered HB-EGF, and 5) increased shedding of HB-EGF after 5-HT treatment. A role for TACE as the sheddase was supported by: 1) co-immunoprecipitation of HB-EGF and TACE, 2) attenuation of 5-HT$_{2A}$-induced ERK phosphorylation by several MMP inhibitors, 3) attenuation of ERK phosphorylation by TACE siRNA, and 4) 5-HT$_{2A}$-induced activation of TACE. The significance of these effects is underscored by the attenuation of 5-HT induced proliferation by siRNA against TACE.

Previous work has demonstrated that metalloproteinases play a role in transactivation of EGFRs in several cell lines by a small panel of GPCRs (8). Generally, those studies have been performed in transfected cell models and have mapped the effects of the transactivation on invasion. Additionally, the roles of metalloproteinases in EGFR transactivation and ERK phosphorylation vary dramatically depending upon the cell type and GPCR being studied (8, 13, 37). Our work clearly demonstrates that TACE can be activated by the 5-HT$_{2A}$ receptor in native renal mesangial cells and that this activation results in shedding of HB-EGF, activation of the EGFR, and initiation of a signaling cascade that results in increased cell proliferation.

HB-EGF shedding is known to contribute to mesenchymal

FIGURE 8. ADAM17/TACE is necessary for 5-HT$_{2A}$-induced increase in DNA content of human mesangial cells. Csi and TACEsi human mesangial cells were treated with vehicle (control) or with 1 or 10 $\mu$M 5-HT in 1% serum-containing medium for 6 days. DNA content was used as a surrogate for cell numbers, as assessed using a fluorescent DNA dye, and expressed as relative fluorescence units (RFU). As positive control we used regular growth medium that contains 17% serum (GM). Results are presented as mean $\pm$ S.E. n = 3; *, p < 0.05 versus control.
cell migration during heart (38) and lung development (39). Accumulation of HB-EGF has been shown in focal lesions in experimental models of focal glomerular sclerosis (38), in the kidney of streptozotocin-induced diabetic rats (40), and in rat kidney after acute injury (41), supporting its role in proliferation and its possible involvement in various states of kidney injury. Angiotensin II has been shown to induce HB-EGF shedding in glomerular mesangial cells through a mechanism that is sensitive to MMP inhibitors; however, the identity of the sheddase was not determined (42).

Although a number of shed ligands, including amphiregulin, betacellulin, TGFrα, epigen, and epiregulin, can bind to and activate EGFRs, we were able to narrow the list of shed growth factors involved in 5-HT-induced ERK phosphorylation using heparin, neutralizing anti-HB-EGF antibody, and the diaphetoxin analogue CM197. Although heparin markedly attenuated 5-HT-induced ERK phosphorylation (Fig. 2A), CM197 or neutralizing antibody against HB-EGF was able to inhibit the 5-HT-evoked ERK activation by ~50% (Fig. 2B and C). Because neutralizing antibodies against other shed growth factors had no effect, the attenuation induced by anti-HB-EGF antibodies suggests that the effects of the antibody are specific. The fact that this antibody was not able to completely inhibit the effect of either 5-HT or exogenous HB-EGF is consistent with the observations of others (43). A fraction of HB-EGF might be in a conformation that obscures key antibody recognition sites or may be protected from antibody sequestration through binding to the cell glycocalyx. We cannot rule out the alternative explanation that shed ligands other than HB-EGF are involved in EGFR transactivation by the 5-HT\textsubscript{2A} receptor, although this appears unlikely in light of the lack of effectiveness of other neutralizing antibodies against known EGFR ligands.

Metalloproteinase activation has been implicated in a number of important processes such as Helicobacter pylori-induced pathophysiology (37), cancer cell motility (44), cardiac hypertrophy (45), neovascularization (46), and cytosic fibrosis (47). Some of those effects could be mediated through shedding of tethered growth factors. Specific metalloproteinases are now starting to be linked with specific cellular and physiological processes. For example, ADAM12 activity has been implicated in the development of cardiac hypertrophy in adult mice (34). ADAM15 may have an essential role in neovascularization in a mouse model of retinopathy (48). TACE/ADAM17 may play important roles in polycystic kidney disease (49). In embryonic cells derived from knock-out mice, ADAM10 and ADAM17 were shown to be the major contributors to the EGFR-like growth factor shedding (50). Based on recently accumulating evidence, it seems that these two enzymes play significant roles in cleaving EGF receptor ligands. However, analysis of tissue-specific activation of metalloenzymes is necessary to reveal whether other ADAMs have a role in specific physiological and/or pathophysiological processes. In the current work, we provide converging lines of evidence that implicate mesangial cell TACE in HB-EGF shedding, EGFR transactivation and ERK activation and proliferation. The functional importance of TACE is underscored by the suppression of both basal and 5-HT-induced proliferation in mesangial cells.

In summary, our data show that 5-HT induced ERK phosphorylation is dependent on ADAM17/TACE enzyme activation and subsequent HB-EGF shedding and EGFR activation in mesangial cells. Because increased DNA mass is a functional consequence of this pathway, TACE might play important roles in untransformed cells in which EGFR transactivation regulates cell growth, proliferation, or mitogenesis. With specific reference to renal mesangial cells, because TACE might contribute to proliferation of mesangial cells, it could participate either in the development of proliferative nephropathy and sclerosis in vivo or in recovery from acute renal glomerular injury. This possibility will require further validation.

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