Serotonin 5-HT$_{2A}$ receptor induces TGF-$\beta$1 expression in mesangial cells via ERK: proliferative and fibrotic signals

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Grewal, J asjit S., Yurii V. Mukhin, Maria N. Garnovskaya, J ohn R. Raymond, and Eddie L. Greene. Serotonin 5-HT$_{2A}$ receptor induces TGF-$\beta$1 expression in mesangial cells via ERK: proliferative and fibrotic signals. Am. J. Physiol. 276 (Renal Physiol. 45): F922–F930, 1999.—We examined the links between fibrotic and proliferative pathways for the 5-HT$_{2A}$ receptor in rat mesangial cells. Serotonin (5-hydroxytryptamine, 5-HT) induced transforming growth factor-$\beta$1 (TGF-$\beta$1) mRNA in a concentration-dependent (peak at 30 nM 5-HT) and time-dependent fashion. For 10 nM 5-HT, the effect was noticeable at 1 h and maximal by 6 h. Inhibition of 1) protein kinase C (PKC), 2) mitogen- and extracellular signal-regulated kinase kinase (MEK1) with 2'-amino-3'-methoxyflavone (PD-90859), and 3) extracellular signal-regulated kinase (ERK) with apigenin attenuated this effect. The effect was blocked by antioxidants, N-acetyl-L-cysteine (NAC) and a-lipoic acid, and mimicked by direct application of H$_2$O$_2$. TGF-$\beta$1 mRNA induction was also blocked by diphenylethionononid and 4-(2-aminoethyl)-benzenesulfonfluryl fluoride, which inhibit NAD(P)H oxidase, a source of oxidants. 5-HT increased the amount of TGF-$\beta$1 protein, validating the mRNA studies and demonstrating that 5-HT potently activates ERK and induces TGF-$\beta$1 mRNA and protein in mesangial cells. Mapping studies strongly supported relative positions of the components of the signaling cascade as follows: 5-HT$_{2A}$ receptor → PKC → NAD(P)H oxidase/reactive oxygen species → MEK → ERK → TGF-$\beta$1 mRNA. These studies demonstrate that mitogenic signaling components (PKC, MEK, and oxidants) are directly linked to the regulation of TGF-$\beta$1, a key mediator of fibrosis. Thus a single stimulus can direct both proliferative and fibrotic signals in renal mesangial cells.

proliferation; fibrosis; mesangial cell; serotonin; transforming growth factor

GLOMERULAR MESANGIAL CELLS have critical roles in the structural integrity and ultrafiltration functions of the kidney. Their morphological localization juxtaposed to the vascular compartment renders them susceptible to a number of vasoactive substances (1, 10), including constrictors like angiotensin II, arginine vasopressin, thromboxane, and serotonin (5-hydroxytryptamine, 5-HT) (16, 22, 37) and relaxants such as dopamine and PGE$_2$ (23). These substances may play key roles in regulating glomerular functions through their actions on mesangial cells. Mesangial cells are normally quiescent, but when activated can proliferate excessively and/or lead to alterations in the amount and composition of extracellular matrix (ECM) in the renal glomerulus. Because early proliferation and subsequent fibrosis are hallmarks of nearly all forms of progressive glomerular diseases (1, 10), an understanding of how various vasoactive substances affect glomerular cell proliferation and ECM homeostasis might yield valuable insight into the common pathways that lead to irreversible renal disease.

Recent studies have implicated mitogen-activated protein kinases (MAPK) of the ERK ("extracellular signal-regulated kinase") family (7) in the pathogenesis of proliferative glomerulonephritis, in experimental antiglomerular basement membrane glomerulonephritis (3) and in human renal cell carcinoma (26). The ERKs are activated by dual-specificity (threonine and tyrosine) protein kinases called MAPKK/MEK. When phosphorylated, the ERKs are capable in turn of phosphorylating a variety of diverse targets such as effector kinases and transcription factors, thereby regulating the expression of different genes associated with mitogenesis (40). Thus there is ample rationale for implicating mesangial cell ERKs as participants in the early proliferative phase of glomerular injury.

In later phases of glomerular disease, abnormal ECM metabolism results in an increased accumulation of ECM and in alteration of matrix composition. Although many mitogens can stimulate production and/or decrease degradation of ECM, the signaling pathways involved in these processes remain obscure. A key signaling molecule prototypically associated with fibrotic processes in many tissues including the renal glomerulus is transforming growth factor-$\beta$ (TGF-$\beta$). TGF-$\beta$ is an important member of a large superfamily of pleiotropic cytokines that play crucial roles during embryonic development, ECM protein synthesis, immune system turnover, cell proliferation, and apoptosis (15, 21, 36). Of several distinct isoforms of TGF-$\beta$, TGF-$\beta$1 is the most abundant. Increased TGF-$\beta$1 mRNA and/or protein levels have been consistently demonstrated in various animal models of glomerulosclerosis (17, 45) and in progressive renal disease in humans (4, 35). Although the cell type responsible for TGF-$\beta$1 production in the glomerulus has not yet been identified, mesangial cells may be one of the major sources. In addition to autoinduction of its own production (14, 42), TGF-$\beta$1 regulates the expression of cellular reactive oxygen species (ROS), which have been implicated in fibrotic processes (31).

Despite their recognized importance in the development of progressive chronic renal diseases, the precise links between the mechanisms of glomerular mesan-
gial cell proliferation and glomerular fibrosis are poorly understood. In the current study, we treated rat mesangial cells with 5-HT to examine its effects on TGF-β1 metabolism and to test the hypothesis that ERKs regulate the expression of TGF-β1. Mesangial cells possess a 5-HT2A receptor (12, 25), which was recently linked to the production of TGF-β1 and collagen through a protein kinase C (PKC)-dependent mechanism (20). Because 5-HT, which is mitogenic, has also been linked to the production of TGF-β1 and to fibrosis in other cell types, it served as an ideal test substance with which to study the potential links between proliferative and fibrotic signals in mesangial cells.

MATERIALS AND METHODS

Chemicals. MaxiScript in vitro transcription kit and RPA II Kit was obtained from Ambion (Austin, TX). 2'-Amino-3'-methoxyflavone (PD-98059), 4-4-fluorophenyl-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB-203580), apigenin, and bisindolylmaleimide (GF-109203) were from Calbiochem (San Diego, CA). [α-32P]UTP (800 Ci/mmol) was from NEN Life Science Products (Boston, MA). The bioactive form of recombinant human TGF-β1, anti-TGF-β1 antibody and Quanti kinetic human TGF-β1 immunoassay kit were from R&D Systems (Minneapolis, ME). Phospho-MAPK (p44/p42) antibody, phospho-SEK1/MKK4 (Thr223) antibody, alkaline phosphatase (AP)-linked rabbit IgG secondary antibody, and CDP Star substrate were purchased from Amersham Biosciences (Arlington Heights, IL). AP-conjugated donkey anti-chicken IgY(H+L) secondary antibody was obtained from Jackson Immunoresearch (West Grove, PA). Precast 4–20% Tris-glycine gradient minigels were bought from Novex (San Diego, CA). All other chemical and reagents were obtained from Sigma (St. Louis, MO).

Cell culture. Primary mesangial cell cultures were raised from glomeruli isolated by sieving the kidneys of 75- to 150-g male Rattus norvegicus Sprague-Dawley rat as described previously (12, 37). Cells were cultured in RPMI-1640 [GIBCO-BRL (Life Technologies), Gaithersburg, MD) supplemented with 20% FBS and 100 U/ml penicillin and 100 µg/ml streptomycin, then incubated at 37°C in a humidified atmosphere of 95% air-5% CO2. Cells were identified as mesangial cells by their characteristic morphology and contractile properties. Cells from passages 6–16 were used in all experiments. Quiescence was induced by transferring the 60–70% confluent cell cultures to RPMI-1640 [GIBCO-BRL (Life Technologies), Gaithersburg, MD) supplemented with 0.5% BSA, 100 U/ml penicillin, and 100 µg/ml streptomycin for 48 h prior to the treatments with various chemicals.

TGF-β Western immunoblotting assay. Equal numbers of quiescent cells (~2 × 105 cells) in 12-well culture plates were treated with various agonists for 10 min, whereas antagonist treatments were carried out for 30 min prior to agonist treatments. Cells were kept at 37°C in an incubator under 5% CO2 atmosphere during all of the treatments. After aspirating the medium, cells were lysed and scraped in 100 µl of Laemmli buffer. Cells were sonicated for 30 s to shear DNA and to reduce viscosity. Samples were heated to 95–100°C in boiling water for 5 min and then shifted to ice. After briefly centrifuging, 20 µl of each sample was loaded on to 4–20% Tris-glycine gradient mini-precast gels for electrophoresis. Proteins were then electrotransferred onto Immobilon-P membranes. Membranes were blocked with blocking buffer (5% nonfat milk in PBS and 0.1% Tween-20) for 1 h before incubating overnight at 4°C with primary antibody (1:1,000 dilution). The membranes were then washed three times with blocking buffer and incubated further for 1 h with alkaline phosphatase-linked anti-rabbit IgG secondary antibody (1:1,000 dilution), except for the TGF-β1 protein assay in which case an AP-conjugated donkey anti-chicken IgY(H+L) secondary antibody was used. After being washed three times with blocking buffer and three times with wash buffer (PBS, 0.1% Tween-20), membranes were incubated for 5 min at room temperature with CDP Star substrate (1:500 dilution). Membranes were then soaked dry between the folds of filter papers and exposed to Kodak X-Omat AR film for 10 s to 15 min and quantified using a GS-670 densitometer and Molecular Analyst software (Bio-Rad, Hercules, CA).

In vitro transcription. The wild-type TGF-β1 sequence in pBluescript II KS+ plasmid, pRTGFb1 (American Type Culture Collection, catalog no. 63197; DNA sequence accession no. X52498) was linearized with BamH I. To get a 245-nt riboprobe, in vitro transcription was performed using T3 DNA-directed RNA polymerase. In brief, 32P-labeled anti-sense mRNA was transcribed using [α-32P]UTP (800 Ci/mmole), RNasin, nucleotides, and buffer conditions as described in the Ambion MaxiScript manual. Internal standards of β-actin or GAPDH riboprobe of low specific activities were transcribed using pTRIPLEScript and higher concentrations (0.4 mM) of cold UTP. On completion of the transcription reaction, the template DNA was removed by adding RNase-free DNase I. The labeled transcripts were then purified by phenol/chloroform extractions and ethanol precipitation. Sizes of the transcripts were verified by electrophoresing samples through denaturing 1.2% agarose/formaldehyde gels.

RNA isolation. Total RNA was isolated by a single-step method (5) using acid guanidium thiocyanate-phenol-chloroform extraction from 80% confluent cells from at least one 100-mm dish. The aqueous phase containing RNA was transferred to a new Eppendorf tube, and RNA was precipitated with 0.7 vol of isopropanol. The RNA pellets were then washed with 70% ethanol to remove salts and isopropanol. Purified RNA was air dried and reconstituted in RNase-free distilled water and stored at −80°C until used.

RNase protection assay. RNase protection assay (RPA) was carried out following the manufacturer’s instructions provided with the Ambion RPA II kit. In brief, 20 µg of sample RNA was hybridized with 50 × 106 cpm of antisense riboprobe in hybridization buffer [80% deionized formamide/100 mM sodium citrate (pH 6.4), 300 mM sodium acetate (pH 6.4), and 1 mM EDTA] for 16–20 h at 48°C. After hybridization, excess single-strand riboprobe and the unhybridized portion of sample RNA was digested away with RNAses A and T1 cocktail (Solution Bx, Ambion) supplemented with 1% blue coprecipitant solution (Ambion) to visualize the pellet. The reaction mixture was incubated at 37°C for 30 min. The RNases were inactivated, and protected double-stranded RNA was precipitated in a single step by adding Solution Dx (Ambion) and incubating at −20°C for 20 min. Samples were then centrifuged for 15 min, and the pellets were air dried and reconstituted in 8 µl of gel loading buffer (8% sucrose, 0.025% bromphenol blue, 0.025% xylene cyanid). The protected segments were isolated on a 5% native polyacrylamide gel. Gels were dried and exposed to Kodak X-Omat AR films for 4–16 h. β-Actin or GAPDH was used to normalize the mRNA loading on the gel. Dried gels were also exposed to a phosphorimagery intensifying screen for the same period for further analysis by the Storm 860 imaging system (Molecular Dynamics, Sunnyvale, CA). Densitometric analysis was performed using ImageQuant supplied by the same manufacturer.

Potentiometric measurement of cellular reduction rate. We used a modified Cytosensor microphysiometer to probe the
redox state of mesangial cells by an electrochemical potentiometric means. We previously used the microphysiometer fitted with silicon sensors to measure proton efflux in mesangial cells (12). In the current studies, we used custom modified gold electrodes (Molecular Devices, Sunnyvale, CA) and an extracellular solution containing ferricyanide and ferrocyanide. Menadione/menadiol was added to the perfusate as a shuttle for carrying electrons across the plasma membrane so that the ability to reduce iron could be measured. The procedure has been validated for several cell types and described in detail recently (29). Cells were grown and maintained as previously described (12). Cells (2.5 × 10^6) were seeded onto polycarbonate inserts (Costar, Cambridge, MA) the night prior to experimentation. After attachment, they were incubated in serum-free Ham’s F-12 medium supplemented with 0.5% BSA and antibiotics until the following day, when they were placed into microphysiometer chambers (12, 29). Prior to each experiment, the chambers and electrodes were washed with distilled water and equilibrated with redox medium consisting of PBS (145 mM Na+, 4 mM KCl, 1 mM Mg2+, 1 mM Ca2+, 143 mM Cl-, and 10 mM phosphate, pH 7.4) supplemented with 10 mM glucose, 20 mM HEPES, 1 ng/mL of endotoxin-free BSA, and 100 µM each of ferricyanide and ferrocyanide. Once loaded, the chambers were maintained at 37°C and perfused with redox medium. The microphysiometer was calibrated to 0 V during perfusion in the absence of menadione/menadiol. Following calibration, measurements were made by switching the perfusate to redox media containing 10 µM menadione. Cells were perfused with menadione-containing medium, then switched to identical medium (to control for valve-switching artifacts) or medium containing test compounds. The chambers were perfused for 58 s, then flow was stopped for 32 s during each 90-s pump cycles. The rate of change in the potential was measured during seconds 20–30 of the stop-flow period. The long delay between stopping perfusion and the reduction rate measurement was needed, because the gold electrodes do not equilibrate with the extracellular ferri/ferrocyanide redox pair as rapidly as the standard pH-sensing electrodes equilibrate with protons (29). Data are presented as percentage change from basal values, which were calculated as the mean values of five consecutive readings taken prior to the readings taken with the test compounds.

Quantitation of activated TGF-β1 concentrations. The TGF-β1 quantitation assay was carried out according to the manufacturer’s instructions (R&D Systems). In brief, 80% and 50% confluent quiescent mesangial cells in 6-well plates were treated with 5-HT and/or the MEK inhibitor PD-98059 for 6 and 24 h. Experiments were also performed in which cells were pretreated with PD-98059 for 1 h, followed by stimulation with 5-HT for 6 and 24 h. To activate latent TGF-β1, the cell culture supernatants were treated with 0.2 vol of 1 N HCl for 10 min at room temperature. Neutralization (to pH 7.2–7.6) of the acidified sample was achieved by adding 0.2 vol of 1.2 N NaOH/0.5 M HEPES solution. Volumes of 200 µl of standard or activated samples were added per well to a 96-well microplate coated with soluble TGF-β1 receptor type II. The microplate was then incubated at room temperature for 3 h. After three washings with wash buffer (0.05% Tween-20 in 1× PBS), 200 µl of horseradish peroxidase-conjugated polyclonal anti-TGF-β1 antibody (1:1,000 dilution) was added to each well and incubated at room temperature for further 90 min. A volume of 200 µl of substrate solution (1:1 mixture of H2O2 and the chromogen tetramethylbenzidine solution) was added to each well after three washings with wash buffer. The reaction was stopped by adding 50 µl of 2 N sulfuric acid to each well. The optical density of each well was determined within 30 min using a microplate reader (Titertek Multiscan MCC/340; ICN, Costa Mesa, CA) set to take dual endpoint readings at 450 nm and a reference wavelength of 540 nm. The standard curve was generated using four parameter logistic curve fit, and from this curve concentrations of activated TGF-β1 in various samples were determined.

Phospho-ERK immunoblots. ERK phosphorylation was assessed using a phosphorylation state-specific ERK antibody (New England Biolabs) which specifically recognizes Tyr204-phosphorylated (but not nonphosphorylated) ERK-1 and ERK-2. The phoso-MAPK antibody was used at 1:1,000 dilution, whereas the control antibody, which recognizes equally well the phosphorylated and nonphosphorylated MAPK, was used at 1:500 dilution per the manufacturer’s recommendations. After treatment, cells were scraped into Laemml buffer, boiled for 3 min, and subjected to SDS-PAGE under reducing conditions with 4–20% precast gels (Novex). After semi-dry transfer to polyvinylidine difluoride membranes, the membranes were blocked with a BLOTTO buffer (5% defatted dried milk in 10 mM Tris, 150 mM NaCl, and 1% Tween-20, pH 8.0). The membranes were incubated overnight with the BLOTTO containing the phoso-MAPK antibody (at 1:1,000 dilution). The membranes were washed, then exposed to goat anti-rabbit alkaline phosphatase-conjugated IgG (1:1,000) in BLOTTO for 1 h, then washed again. Immunoreactive bands were visualized by a chemiluminescent method (CDP Star, New England Biolabs) using preflushed Kodak XAR film.

RESULTS

TGF-β1 induction. We first tested for the induction of TGF-β1 mRNA by 5-HT by ribonuclease protection assay. Cells treated with 5-HT showed both concentration- and time-dependent increases in TGF-β1 mRNA levels. 5-HT induced detectable increases in TGF-β1 mRNA at 2 h, which were maximal after 6 h of treatment (Fig. 1A). At 30 nM, 5-HT induced nearly peak stimulation (Fig. 1B), which coincides closely with the concentration of 5-HT required for maximal stimulation of the ERKs (data not shown). The EC50 concentrations for 5-HT activation of ERK and increased TGF-β1 mRNA were both ~10 nM, suggesting a possible close linkage between their signal transduction pathways. Although Nebigil et al. (25) have identified and sequenced a 5-HT2A receptor in mesangial cells, we verified that the 5-HT2A receptor was responsible for TGF-β1 mRNA induction. Ketanserin tartrate, a selective 5-HT2A/5-HT2C receptor inhibitor, blocked the induction of TGF-β1 (2.5-Dimethoxy-4-iodoamphetamine hydrochloride (DOI HCl), a potent and selective 5-HT2A/5-HT2C receptor agonist, increased the induction of TGF-β1 (Fig. 1C). Because the 5-HT2C receptor is not present in these cells (12, 25), the results are consistent with activation of the 5-HT2A Receptor.

Lack of involvement of pertussis toxin-sensitive G protein and involvement of PKC. Pertussis toxin was able to block the induction of TGF-β1 mRNA by 5-HT only slightly (Fig. 2A) under conditions previously shown to cause ADP-ribosylation of nearly all mesangial cell G1o proteins (12). As the 5-HT2A receptor is nearly always coupled to activation of PKC, we tested the role for PKC in the increase of TGF-β1 mRNA in
mesangial cells. Phorbol 12-myristate, 13-acetate (PMA), a direct PKC activator, was used to establish that TGF-β1 mRNA increases can be mediated via PKC activation. PMA treatment (1 µM for 6 h) increased TGF-β1 mRNA. Prolonged treatment with PMA for 24 h, which exhausts cells of PKC by accelerating its degradation, blocked the induction of TGF-β1 mRNA by 5-HT (Fig. 2B). Similarly, apigenin (4,5,7-trihydroxyflavone) known to inhibit MAPK, also blocked TGF-β1 mRNA induction (Fig. 2C). To test the specificity of the inhibition of TGF-β1 mRNA by the MEK inhibitor PD-98059, and by apigenin, we studied the effects of SB-203580, a highly specific inhibitor of cell-permeable serine/threonine kinase inhibitor, also blocked TGF-β1 mRNA induction by 5-HT (Fig. 2B).

Involvement of ERKs and MEK1. One hour of pre-treatment with the MEK inhibitor PD-98059, which binds to the inactive form of mitogen- and extracellular signal-regulated kinase kinase (MEK1) and prevents its activation, blocked the stimulation of TGF-β1 mRNA nearly to the basal level (Fig. 2C). Similarly, apigenin (4,5,7-trihydroxyflavone) known to inhibit MAPK, also blocked TGF-β1 mRNA induction (Fig. 2C). To test the specificity of the inhibition of TGF-β1 mRNA by the MEK inhibitor PD-98059, and by apigenin, we studied the effects of SB-203580, a highly specific inhibitor of
Acetyl-L-cysteine (NAC) blocked the stimulation of TGF-β mRNA. A thiol-based antioxidant increased TGF-β in the effects of two antioxidants on the ability of 5-HT to block induction of TGF-β mRNA. Because inhibition of MEK and ERK MAPKs in mesangial and vascular smooth muscle cells (32, 41, 44), because inhibition of MEK and ERK blocked induction of TGF-β1 mRNA by 5-HT, we tested the effects of two antioxidants on the ability of 5-HT to increase TGF-β1 mRNA. A thiol-based antioxidant (N-acetyl-L-cysteine, NAC) blocked the stimulation of TGF-β1 mRNA nearly to the basal level (Fig. 3A), suggesting that the generation of ROS is critical in the signaling cascade that upregulates TGF-β1 mRNA. α-Lipoic acid (DL-thioctic acid) (33), a broad range antioxidant, also significantly reduced the TGF-β1 mRNA induction by 5-HT compared with the basal level (Fig. 3A). Thus two chemically distinct antioxidants blocked the upregulation of TGF-β1 mRNA by 5-HT. If 5-HT induces TGF-β1 mRNA through the generation of oxidant molecules, then one would expect that application of exogenous ROS should mimic that effect. Accordingly, various concentrations (300 pM to 1 µM) of H2O2 were applied to mesangial cells, and after 6 h, the cells were harvested for analysis by ribonuclease protection assay. Because H2O2 is a labile and reactive molecule, the H2O2 was replenished every hour. The treatment of cells with H2O2 resulted in upregulation of TGF-β1 mRNA at a threshold level of 1 µM H2O2 (Fig. 3B). H2O2 at 1 µM increased TGF-β1 mRNA to a level that was equivalent to that induced by 5-HT. These studies support a role for ROS as second messengers in the pathway from the 5-HT2A receptor in mesangial cells, which results in increased expression of TGF-β1 mRNA.

NAD(P)H oxidase enzymes have been increasingly implicated as participants in the generation of ROS in various cell types including the mesangium (32, 41). Thus we tested the effects of two chemical inhibitors of NAD(P)H oxidase on the induction of TGF-β1 mRNA by 5-HT. Diphenyleneiodonium chloride (DPI), which interferes with the flavin binding site of NAD(P)H oxidase, and 4-(2-aminoethyl)-benzenesulfonyl fluoride HCl (AEBSF), an irreversible serine protease inhibitor that prevents the activation of NADPH oxidase by preventing assembly of its subunits (8), were also able to block TGF-β1 message induction by 5-HT (Fig. 3C).

If 5-HT increases TGF-β1 mRNA through the generation of ROS, then we should be able to detect rapid changes in cellular redox potential induced by 5-HT. To address this issue, we used a redox shuttle system consisting of ferrous and ferric iron, and the shuttle compound, menadione, to facilitate extracellular detection of changes in intracellular redox states. Figure 4A shows representative tracings of cell monolayers exposed for five pump cycles to 1 µM 5-HT in the presence or the absence of cells. In chambers with cells, 1 µM 5-HT increased the reduction rate by 13 ± 2% (n = 5), and 5 µM 5-HT increased the reduction rate by 17 ± 2% (n = 9). In empty chambers, 5-HT elicited no changes in the reducing rate, ruling out simple chemical interactions of 5-HT with the redox shuttle system components. In chambers exposed to perfusate not containing the electron shuttle, menadione, 5-HT did not elicit an
increase in reduction rate, confirming that the shuttle is necessary for reducing equivalents to be transferred from the intracellular environment to the extracellular space (Fig. 4B, solid bar). The selective 5-HT2A receptor partial agonist DOI increased the reduction rate by 11.62% (Fig. 4A, n = 6). Application of sodium azide, which increases cellular oxidative stress by inhibiting the mitochondrial electron transport chain, also increased the reduction rate by 11.63% (n = 3), demonstrating that a receptor-independent oxidative stimulus also increases the reduction rate. To demonstrate that the increase in reduction rate is not a peculiarity of the 5-HT2A receptor, we treated cells with 10 µM ATP to stimulate another endogenous Gq-coupled purinergic receptor. ATP increased the reduction rate by 8.4% (n = 6). The effect of ATP is not simply due to increased energy supplies, as the intracellular concentration of ATP in most cells (1–5 mM) is in vast excess to the amount that we applied to the mesangial cells.

Mapping of the relative positions of PKC, NAD(P)H oxidase, ROS, and ERK in the TGF-β1 cascade. Figure 5 shows the results of mapping studies in which TGF-β1 production was stimulated by PMA. PMA increased TGF-β1 mRNA by ~1.5-fold, and this increase could be attenuated by at least 60% by PD-98059, DPI, or NAC. Those studies suggest the placement of MEK and NAD(P)H oxidase downstream of PKC in the TGF-β1 cascade. To confirm this placement, we examined the effects of PD-98059, DPI, or NAC to inhibit the phosphorylation of ERK induced by treatment for 10 min with 1 µM PMA. PMA increased the phosphorylation of ERK to >500% of control levels, and this effect was almost completely inhibited by PD-98059, DPI, or NAC.

5-HT induced TGF-β1 protein quantitation. Upregulation of most mRNA species is only important if there is a concomitant increase in the quantities and/or activities of the proteins that they encode. To determine whether TGF-β1 mRNA induction is accompanied by a subsequent increase in TGF-β1 protein, we performed a quantitative sandwich ELISA. Cells treated with 5-HT showed activated TGF-β1 protein more than six times the basal level. The increase in the active form of TGF-β1 protein was blocked by the MEK inhibitor, PD-98059 (Fig. 6A). The results were similar in cells that were 50% and 80% confluent at 6- and 24-h incubation with 5-HT. Similar results were seen with an immunoblot of the active form of TGF-β (Fig. 6B).

TGF-β1 autinduction of TGF-β1 mRNA. In some cells, TGF-β1 has been shown to support its own induction. We tested the effects of TGF-β1 on TGF-β1 mRNA in mesangial cells and found that there was
concentration-dependent increase in TGF-\(\beta\)1 mRNA (Fig. 7). Thus, in mesangial cells, TGF-\(\beta\)1 has the potential to autoinduce its own expression.

DISCUSSION

The molecular mechanisms underlying the abnormal proliferation and fibrosis in chronic renal diseases are only now being unraveled. It is very likely that dysregulation of ERKs is involved in the early proliferative phase of chronic renal diseases, whereas TGF-\(\beta\) is likely involved in the later fibrotic phase. Nevertheless, the relationship between those two phases or their molecular mediators has remained elusive. What is new about the current work is that we have described a clear relationship between ERK activity and TGF-\(\beta\)1 mRNA levels and protein in mesangial cells. In addition, our data also suggest that ROS play a role in the regulation of TGF-\(\beta\)1 expression by 5-HT. These findings are particularly interesting in light of prior evidence that has implicated ROS in the activation of ERKs in mesangial cells (44).

Our work was facilitated by the use of a single substance, 5-HT, which has been linked both to abnormal cellular proliferation and to fibrotic diseases. Serotonin (i.e., 5-HT) is known as a potent mitogen in many cells, activating cell growth through both pertussis toxin-sensitive (2, 34) and -insensitive G-proteins (13). It has also been implicated in fibrotic disorders, like the retroperitoneal fibrosis associated with methysergide (18), carcinoid heart disease (43), and pulmonary hypertension/aortic valve disease associated with fenfluramine and phentermine (6, 19). 5-HT was also recently shown to increase the expression of collagen in mesangial cells through the actions of PKC and TGF-\(\beta\)1, with TGF-\(\beta\)1 residing downstream of PKC (20). The potential significance of these observations is enhanced by an extensive older literature in which 5-HT has been implicated in various forms of nephropathy (9, 24).

Previous evidence has supported the possibility that various effects of 5-HT are mediated by the cytokine, TGF-\(\beta\). Zhang et al. (46) demonstrated in the isolated Aplysia ganglion that TGF-\(\beta\) mediates the effects of 5-HT on long-term facilitation. Similarly, Pousset et al. (28) showed in rat primary hippocampal astrocytes that 100 pM 5-HT induced TGF-\(\beta\)1 mRNA in vitro. While this report was under review, Masaya et al. (20) linked 5-HT to production TGF-\(\beta\)1 and type IV collagen in mesangial cells. In the present studies, we have demonstrated that rat mesangial cells respond to 5-HT treatment (5-HT\(_{2A}\) receptor subtype) in a concentration- and treatment time-dependent manner by

![Fig. 7. Autoinduction of TGF-\(\beta\)1 mRNA by TGF-\(\beta\). ELISA showed that the bioactive form of recombinant human TGF-\(\beta\)1 protein was able to induce TGF-\(\beta\)1 mRNA at 6 h at concentrations as low as 0.5 ng/ml. Results are representative of 2 independent experiments. Inset is from representative experiments. *Significance level of P < 0.05 compared with control.](http://ajprenal.physiology.org/)

![Fig. 8. Proposed schematic of signaling cascade leading from 5-HT\(_{2A}\) receptor to TGF-\(\beta\)1, fibrosis, and proliferation of mesangial cells. This hypothetical scheme is based on work presented in this report and on previous reports from other groups (14, 16, 20, 22, 30, 39). PLC, phospholipase C.](http://ajprenal.physiology.org/)
inducing TGF-β1 mRNA. We further demonstrated that an increase in TGF-β1 mRNA levels is accompanied by increased TGF-β1 protein levels. 5-HT-treated cells showed TGF-β1 protein levels that were as high as six times the basal values, as detected by Western blot and ELISA. This is an essential finding that validates the significance of the increased mRNA levels.

Several groups previously showed that thromboxane (38, 39) increases TGF-β1 through pathways that involve PKC and ROS in mesangial cells, whereas 5-HT increases TGF-β1 through PKC (20). Moreover, it is already known that PKC is a critical intermediate in the activation of proliferation in mesangial cells by the 5-HT₃A receptor (11, 30).

The increase in TGF-β1 mRNA by PMA, a direct activator of PKC, and the blockade of 5-HT-induced increases in TGF-β1 mRNA by PKC inhibitors, supports the involvement of PKC in the regulatory pathway initiated by 5-HT. The main data linking ERK with TGF-β1 induction are provided by the blockade induced by PD-98059, a specific inhibitor of MEK1, and by apigenin, an inhibitor of ERKs. The specificity of the involvement of the ERK type of MAPK was demonstrated by lack of involvement of SAPK/J UNK or of p38 MAPK. These studies link a specific mitogenic cascade (MEK1 → ERK) with a fibrogenic cascade (TGF-β1) in rat mesangial cells.

We tested a potential role for ROS in the induction of TGF-β1 mRNA by 5-HT. Four lines of evidence supported a role for ROS in this process. First, the induction of TGF-β1 mRNA was attenuated by treating cells with two structurally distinct thiol antioxidants (NAC; and the reduced form of α-lipoic acid) prior to stimulation with 5-HT. NAC serves as an antioxidant by directly protecting sulfhydryl groups from oxidation or indirectly by serving as a precursor for the synthesis of glutathione, an abundant endogenous cellular reducing antioxidant, and by recycling other antioxidants (27, 33). α-Lipoic acid is a scavenger of hydroxyl radicals, singlet oxygen, and hypochlorous acid and may exert antioxidant effects by chelation of transition metals (33).

The second line of evidence supporting the involvement of ROS is that direct application of the weak oxidant, H₂O₂, also induced TGF-β1 mRNA to a degree similar to that demonstrated for 5-HT. This effect was seen at a low threshold concentration of 1 μM, suggesting that this effect is not secondary to nonspecific cellular toxicity. The third line of evidence is provided by experiments demonstrating that the induction of TGF-β1 mRNA could be blocked by two structurally distinct chemical inhibitors of NAD(P)H oxidase, which is a major enzymatic generator of cellular oxygen free radicals. Both DPI (a flavin site blocker) and AEBSF [which prevents assembly of the functional NAD(P)H oxidase enzyme] also decreased the TGF-β1 mRNA induction by 5-HT. The fourth line of evidence demonstrated that 5-HT rapidly alters mesangial cell redox as assessed by microphysiometry. Mapping studies strongly supported a relative position of the NAD(P)H oxidase and ROS in the signaling cascade as follows: 5-HT₃A Receptor → PKC → NAD(P)H oxidase/ROS → MEK → ERK → TGF-β1 mRNA. However, because PD-98059, a specific inhibitor of MEK1, completely blocked the phosphorylation of ERK induced by PMA, but only attenuated the increase in TGF-β1 mRNA induced by PMA by ~60% (Fig. 5), it is possible that a PKC-driven, MEK1-independent pathway also exists.

Based on our studies, and those of others (14, 16, 20, 22, 30, 39), we propose a signal transduction model as depicted in Fig. 8.

A final point of interest is the ability of TGF-β1 to induce its own mRNA in mesangial cells. This finding supports the possibility that an initial mitogenic exposure to a substance such as 5-HT could lead to proliferation via direct activation of ERKs and secondarily to generate a sustained fibrotic stimulus by inducing TGF-β1 expression which in turn is able to sustain its own induction. Overall, these studies provide a potential link between the mechanisms that induce proliferation and those that induce and sustain fibrosis in renal mesangial cells.

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REFERENCES


