5-HT$_{2A}$ receptors stimulate mitogen-activated protein kinase via H$_2$O$_2$ generation in rat renal mesangial cells

Eddie L. Greene, Odette Houghton, Georgiann Collinsworth, Maria N. Garnovskaya, Toshio Nagai, Tahir Sajjad, Venugopala Bheemanathini, Jasjit S. Grewal, Richard V. Paul, and John R. Raymond

Nephrology Division, Department of Internal Medicine, Medical University of South Carolina, and Ralph H. Johnson Veterans Affairs Medical Center, Charleston, South Carolina 29425

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
signal-regulated kinase (MEK, the kinase that phosphorylates and activates ERK) and the generation of ROS (19). Therefore, the present studies were performed to establish that 5-HT activates an ERK subtype in rat glomerular mesangial cells and, furthermore, to explore a potential role for ROS in transmitting the signal from 5-HT to the ERK molecules.

MATERIALS AND METHODS

Materials. Drugs and reagents were obtained from the following sources: A-23187, arsenite, buthionine sulfoximine (BSO), diamide, 5-HT, H2O2, α-lipoic acid (reduced form), 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)maleimide (GF-109203X), myelin basic protein, N-acetylcyesteine (NAC), pertussis toxin, phorbol 12-myristate 13-acetate (PMA), cytochrome c, and tert-butyl hydroperoxide from Sigma Chemical (St. Louis, MO); 1-oxade-2-O-methyl-sn-glycerol-3-phosphorylcholine (ET-18-OCH3), 1-(6′-17β-methoxyestradiol, 1,3,5(10)-trien-17-yl)-a-methylamino)hexyl-2,5 pyrrilodine-dione (U-73343) and 1-(6′-(17β-3-methoxyestradiol, 1,3,5(10)-trien-17-yl)-a-methylamino)hexyl-2,5 pyrrilodine-dione (U-73343) from Biomol (Plymouth Meeting, PA); PD-98059 from Calbiochem (San Diego, CA); 2-′,7′-dichlorofluorescin diacetate (DCF-DA) from Molecular Probes (Eugene, OR); 1-(5-isouquinoline-sulfonyl)-2-methylpeperazine and thapsigargin from LC Laboratories (Woburn, MA); and [α-32P]ATP from DuPont-NEN (Boston, MA). Cell culture media, serum, and antibiotics were obtained from Gibco-BRL (Gaithersburg, MD) and culture flasks from Costar (Cambridge, MA). The phospho-MAPK kits were obtained from New England Biolabs (Beverly, MA).

Isolation and primary culture of rat glomerular mesangial cells. Mesangial cells were obtained from cortical sections of kidneys from young 100- to 150-g Sprague-Dawley rats by use of a standard sieving technique (30). Cells were incubated at 37°C in a humidified atmosphere of 95% air-5% CO2 and subcultured every 1–2 wk by trypsinization until pure cultures of mesangial cells were obtained. They were plated at a density of 2–5 × 106 cells/ml in RPMI medium supplemented with 20% FCS and antibiotics (100 U/ml of penicillin and 100 µg/ml of streptomycin). At 48 h before studies, cells were placed in serum-free RPMI medium supplemented with antibiotics. Cells from passages 5–16 were used.

ERK assays. ERK activity was measured in immune complexes with myelin basic protein as the substrate (17). For most experiments, ERK phosphorylation was used as a surrogate for kinase activity. ERK phosphorylation was assessed using a phosphorylation state-specific ERK antibody (New England Biolabs) that specifically recognizes tyrosine-204-phosphorylated (but not nonphosphorylated) ERK1 and ERK2 and does not react with closely related p38 MAPK or J un kinases or stress-activated protein kinases (JNK/ SAPKs). The phospho-ERK antibody was used at 1,000 dilution, whereas the control antibody, which recognizes equally well the phosphorylated and nonphosphorylated ERK, was used at 1:500 dilution per the manufacturer’s recommendations. Blotting and visualization were carried out as previously described (17).

Measurement of superoxide anion production. Superoxide anion (O2·−) production was quantified by the cytochrome c reduction assay (31) with modifications. Briefly, cells were grown to 60–80% confluency in six-well culture plates and starved in serum- and phenol red-free medium for 48 h. The cells were further incubated in 1 ml of serum- and phenol red-free medium containing 200 µM cytochrome c and 1 µM 5-HT or 0.5 µM PMA in the presence or absence of 300 U/ml of superoxide dismutase (SOD) for 60 min at 37°C in a humidified incubator with 5% CO2. Cells were pretreated with inhibitors [50 µM diphenyletheniodide (DPI) or 2 µM GF-109203X] for 30 min before application of 5-HT or PMA. Absorbance of the cell-free supernatant was measured spectrophotometrically at 550 nm. The following equation was used to determine O2·− produced in picomoles

\[ O_2·^{−} \text{pmol/10}^6 \text{cells} = 0.001 \times [A_{550}(\text{without SOD}) - A_{550}(\text{with SOD})] \times 47.6 \]

Measurement of intracellular H2O2 generation. The H2O2-sensitive fluorescent probe DCF-DA was used to assess the generation of intracellular H2O2 (8, 33). Nonfluorescent DCF-DA diffuses through the plasma membrane, where it is subsequently deacetylated enzymatically by cellular esterases to the polar compound 2′,7′-dichlorofluorescein (DCF), which remains trapped in the cell and fluoresces in the presence of intracellular peroxides (H2O2 and lipid hydroperoxides). Cells in monolayer were incubated with Earle's balanced salt solution supplemented with 10 µM DCF-DA and 1% BSA (wt/vol) for 30 min at 37°C. The supernatant was removed and replaced with fresh unsupplemented Earle's solution before stimulation with 5-HT, which was added from a 1,000× stock directly to the Earle's solution before analysis. Relative fluorescence intensity and fluorescent images were obtained over time (0.5–20 min) by laser confocal scanning microscopy (LSMGB-200, Olympus Optical, Tokyo, J. apalan) at an excitation wavelength of 485 nm; emission was measured at a wavelength of 530 nm.

RESULTS

5-HT induces phosphorylation and activation of ERK via the 5-HT2A receptor. Treatment of mesangial cells with 5-HT resulted in an increase in phosphorylation of ERK and in activation of ERK as determined by immunoprecipitation kinase assay in which myelin basic protein was the substrate (Fig. 1A). The phosphorylation and activation of ERK induced by 5-HT were inhibited by the specific 5-HT2A receptor antagonist ketanserin (10 µM) and mimicked by the specific 5-HT2A receptor agonist R-[−]2,5-dimethoxy-4-iodoamphetamine hydrochloride (DOI) (10 µM). Those findings verify that the signal is conveyed by the 5-HT2A receptor that is expressed in rat mesangial cells (16, 28). Figure 1B shows that the coupling of the 5-HT2A receptor to ERK phosphorylation was quite efficient, with an EC50 of 12 ± 7 nM. Notably, this coupling was about one order of magnitude more potent than that of this receptor for hydrolysis of inositol phosphates (−265 nM), a second messenger pathway that is almost universally linked to this receptor subtype (16). The time course (Fig. 1C) also is consistent with that expected for activation of ERK by G protein-coupled receptors, with phosphorylation first being apparent as early as 1 min, peaking at 5–15 min of exposure to 5-HT, and persisting for up to 60 min. Moreover, the signal was also blocked by the mitogen-activated extracellular signal-regulated kinase kinase (MEK1) inhibitor PD-98059, as expected for receptor-activated ERK (Fig. 1B).
Involvement of phospholipase C and classical PKC and lack of involvement of pertussis toxin-sensitive G proteins in the phosphorylation of MAPK by 5-HT. 5-HT2A receptor classically signals by activating phospholipase C (PLC) and phorbol ester-sensitive classical PKC types. This typically occurs through non-pertussis toxin-sensitive G proteins, although we previously showed that in rat mesangial cells the 5-HT2A receptor inhibits cAMP and activates a proton efflux that are mainly sensitive to pertussis toxin (16). The potential involvement of pertussis toxin-sensitive Gi/o proteins was tested by preincubation of cells overnight with pertussis toxin (200 ng/ml). This treatment has previously been shown to greatly attenuate 5-HT2A receptor-inhibited cAMP and stimulation of proton efflux and to nearly completely eliminate the subsequent ADP-ribosylation of Gi/o proteins by pertussis toxin in these cells (16). In the present study, pertussis toxin had no effect on the ability of 5-HT to activate ERK (Fig. 2A), effectively ruling out a substantial role for Gi/o proteins in conveying the signal from the 5-HT2A receptor to ERK.

We previously showed that 5-HT activates PLC-mediated hydrolysis of inositol phosphates (16), whereas others have shown that 5-HT stimulates PKC in mesangial cells (27, 40). The involvement of PLC as a contributor to the activation of ERK was tested by incubating mesangial cells with two PLC inhibitors, ET-18-OCH3 and U-73122 (each at 20 µM), and U-73343, an inert analog of U-73122. Both PLC inhibitors significantly blocked the phosphorylation of ERK by 5-HT, but the inert analog U-73343 had no effect, confirming a role for PLC in this pathway (Fig. 2A).

PLC classically results in the generation of two second messengers, inositol trisphosphate (IP3) and diacylglycerol (DAG). DAG can directly activate classical types of PKC by interacting with its lipid-binding domain, and IP3 can indirectly activate PKC by increasing intracellular Ca2+, which interacts with the PKC Ca2+-binding domain. We therefore tested the potential involvement of classical PKC in the activation of ERK in two ways (Fig. 2B). First, cells were pretreated for 15 min with two PKC inhibitors, H-7 (50 µM) and GF-109203X. Second, cells were pretreated overnight with 1 µM PMA for 18 h to downregulate phorbol ester-sensitive PKC types. Those maneuvers inhibited the ability of 5-HT to phosphorylate ERK. Furthermore, in

Fig. 1. Serotonin (5-HT) activates extracellular signal-regulated kinase (ERK) in rat glomerular mesangial cells. A: cells were treated with test agents for 10 min. Cells treated with antagonist (ketanserin) were preexposed for 5 min before stimulation with 5-HT. ERK phosphorylation was assessed by immunoblot with phosphorylation state-specific antibody (phospho-ERK blot), which was compared with results of blots probed with an ERK antibody that is not sensitive to ERK phosphorylation (ERK blot). Results of an activity assay that measures ability of immunoprecipitated ERK to phosphorylate myelin basic protein (MBP) are also shown (MBP substrate assay). B: time course of ERK phosphorylation after treatment with 1 µM 5-HT (0–60 min). Each experiment was performed ≥3 times in duplicate or triplicate. Error bars, SE. *P < 0.5 vs. vehicle control unless indicated by a spanning bar (A). Reverse Bonferroni correction was used to correct for multiple comparisons.

Fig. 2. Effects of treatment with pertussis toxin, phospholipase C (PLC) inhibitors, and protein kinase C (PKC) inhibitors on 1 µM 5-HT stimulation of ERK phosphorylation. A: ERK phosphorylation was not affected by preincubation overnight with pertussis toxin (200 ng/ml). 5-HT-induced ERK phosphorylation was blunted by 2 inhibitors of PLC (U-73122 and ET-18-OCH3) but not by an inactive analog of U-73122 (U-73343). B: 3 maneuvers that block PKC attenuated 5-HT-induced ERK phosphorylation. Treatments with 500 nM phorbol 12-myristate 13-acetate (PMA; PKC activator) for 10 min also resulted in ERK phosphorylation. Plots are representative of 3–5 separate experiments performed in triplicate. Error bars, SE. *P < 0.5 vs. 5-HT value in presence of vehicle control. Reverse Bonferroni correction was used to correct for multiple comparisons.
data not shown, another specific PKC inhibitor, chelerythrine, also attenuated the phosphorylation of ERK by 5-HT. Those results implicate phorbol ester-sensitive classical PKC as an intermediary between the 5-HT$_{2A}$ receptor and ERK. This hypothesis was supported further by the ability of PMA, a direct activator of PKC, to induce phosphorylation of ERK to a level equivalent to 5-HT. However, because the blockade of ERK activation was not total, it is likely that another signaling intermediate (such as a nonclassical PKC subtype or a tyrosine kinase) may mediate the remainder of the response.

Involvement of ROS in the phosphorylation of MAPK by 5-HT. To establish a potential role for ROS in conveying the stimulation of ERK by the 5-HT$_{2A}$ receptor, we established five criteria that would need to be fulfilled experimentally: 1) antioxidants should attenuate the effects of 5-HT, 2) the effects of 5-HT should be mimicked by direct application of molecules, which generate ROS, 3) a specific enzyme capable of generating ROS should be implicated, 4) there should be evidence of specificity in the actions of ROS on the ERK pathway, and 5) 5-HT should produce measurable amounts of ROS in a time scale similar to that of ERK activation.

To address the first criterion, we treated cells with two structurally distinct antioxidant molecules (the reduced form of α-lipoic acid and NAC) and then with 5-HT. NAC can serve as an antioxidant directly by protecting sulfhydryl groups from oxidation and indirectly by serving as a precursor for the synthesis of glutathione, an abundant endogenous cellular reducing antioxidant (35). α-Lipoic acid functions mainly as a scavenger of hydroxyl radicals, singlet oxygen, and hypochlorous acid. α-Lipoic acid may also exert antioxidant effects by chelation of transition metals. In addition, α-lipoic acid may have indirect antioxidant effects as well, by recycling other antioxidants or increasing cellular levels of glutathione (29, 35). Figure 3A demonstrates that overnight incubation with α-lipoic acid (500 µM) virtually eliminates the ability of 5-HT to activate ERK without affecting basal levels of phospho-ERK. The effect of α-lipoic acid could be reversed by coinubcation with 400 µM racemic BSO, an inhibitor of γ-glutamylcysteine synthase. Because γ-glutamylcysteine synthase is the rate-limiting enzyme in glutathione synthesis, BSO treatment should deplete cells of the important antioxidant glutathione (20). Preincubation with 20 mM NAC for 30 min also markedly blunted the ability of 5-HT to increase the phosphorylation of ERK. Preincubation with 50 mM NAC blunted the response to a similar level, as did overnight treatment with α-lipoic acid (Fig. 3A). These experiments clearly indicate the redox state of the cell as critical in the ability of mesangial cells to respond to treatment with 5-HT.

Moreover, the effect of NAC is not merely due to reduction of extracellular disulfide bonds contained within the 5-HT$_{2A}$ receptor, because the effect of PMA (which works at an intracellular site downstream of the receptor) is blunted by antioxidants as efficiently as is the effect of 5-HT stimulation (see Fig. 5).

If ROS participate in 5-HT$_{2A}$ receptor- and PKC-induced activation of ERK, it would be expected that oxidative stress might also activate ERK. Indeed, depletion of cellular glutathione stores with BSO treatment somewhat increased the basal phosphorylation of ERK in mesangial cells (Fig. 3A), although this effect was not statistically significant. To confirm the effects of oxidant stress on mesangial cell ERK, cells were treated with four substances that have been shown to induce oxidative stress: H$_2$O$_2$ (200 µM), sodium arsenite (400 µM), diamide (500 µM), and tert-butyl hydroperoxide (t-butyl HP) or 400 µM sodium arsenite for 15 min. For some studies, cells were preincubated with BSO, α-lipoic acid, or NAC as described in A. C: concentration-response plot of ERK phosphorylation for 15-min treatments with H$_2$O$_2$. D: time course of ERK phosphorylation for 1 mM H$_2$O$_2$. Error bars, SE. *P < 0.5 vs. 5-HT value in presence of vehicle control. Reverse Bonferroni correction was used to correct for multiple comparisons.
membranes rapidly, where it efficiently oxidizes thiols (23). These three oxidant-generating molecules can interact efficiently with methionine and cysteine residues in proteins. Tert-butyl hydroperoxide is a molecule with oxidative capacity similar to H$_2$O$_2$. Unlike the three other oxidant-generating molecules, tert-butyl hydroperoxide selectively interacts with methionine residues of proteins, presumably because of its bulky butyl group (12). As shown in Fig. 3B, addition of H$_2$O$_2$, diamide, and sodium arsenite, but not tert-butyl hydroperoxide, increased the amount of phospho-ERK present in mesangial cell extracts. The stimulatory effect of H$_2$O$_2$ was markedly diminished in cells pretreated overnight with NAC or α-lipoic acid (Fig. 3B) but was not diminished in cells pretreated with BSO and α-lipoic acid (data not shown).

The stimulation was further characterized for H$_2$O$_2$ as being dependent on time and concentration, being apparent at 3 min, peaking at 15–30 min, and being maintained for ≥90 min. During a 10-min incubation, the stimulation was apparent at 200 mM H$_2$O$_2$, had peak effect at 1 mM, and had lesser effects at 10 and 25 mM (Fig. 3, C and D). Thus oxidant-generating substances mimic the effects of 5-HT on ERK, and this effect is specific for certain oxidant-generating molecules.

We hypothesized that mesangial cell NAD(P)H oxidase was the enzyme responsible for generating the ROS in response to 5-HT (19). Because NAD(P)H oxidase is a multicomponent enzyme (9), we were able to employ four types of pharmacological inhibitors of NAD(P)H oxidase to test its involvement in the activation of ERK by 5-HT. 4′-Hydroxy, 3′-methoxy acetophenone (HMAP) competes with NAD(P)H for a binding site on the oxidase but does not effectively complete an obligate two electron transfer to FAD (11, 34). This transfer to FAD is critical for enzyme activity, in that it is required to convert the obligate two electrons donated from NAD(P)H to a one-electron transfer required from the heme site of the NAD(P)H enzyme to O$_2$ to yield O$_2^·$. Another inhibitor, DPI, blocks FAD binding to the flavin site of the oxidase (11). Phenylarsine oxide (PAO) blocks distal electron transport in the NAD(P)H oxidase (24). Finally, 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) is a serine protease inhibitor that also has been shown to block NAD(P)H oxidase by preventing assembly of the enzyme subunits (13).

Figure 4A shows that HMAP, DPI, and PAO markedly attenuated the activation of ERK by 5-HT. Because ROS could derive from mitochondrial sources and because it is possible that a flavin inhibitor such as DPI or a competitor of NAD(P)H such as HMAP could inhibit mitochondrial electron transport (thereby increasing mitochondrial production of ROS), we tested the effects of three inhibitors of mitochondrial electron transfer on phosphorylation of ERK induced by mitogens. Sodium azide, a blocker of complex IV, had no effect on ERK phosphorylation (Fig. 4A), making it unlikely that the ROS derive from the mitochondria. We also tested blockers of complex I (rotenone) and complex III (antimycin), neither of which had an effect on 5-HT-stimulated ERK phosphorylation (data not shown). The effects of PAO (100 µM) could be partially reversed by coinoculation with British anti-Lewisite (BAL) as has been shown to occur with purified NAD(P)H oxidase components (24). This feature is an important step in validating the interaction of PAO with the NAD(P)H oxidase and not some other signaling enzyme such as a tyrosine phosphatase. We did not expect that BAL would completely reverse the effects of PAO, because the two compounds are thought to compete for a single binding site within the NAD(P)H oxidase (24).

AEBSF effectively blocked the activation of ERK by 5-HT (Fig. 4B). A series of protease inhibitors with varying degrees of structural similarities to AEBSF was also tested for the ability to inhibit the activation of ERK by 5-HT. As shown in Fig. 4B, 4-(2-n-methylamino)benzenesulfonyl fluoride (AEBSF), 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), or other serine protease inhibitors with varying degrees of structural similarities to AEBSF for 15 min before treatment with 5-HT or vehicle. Attenuation induced by PAO was reversed by coinoculation with British anti-Lewisite (BAL), as would be expected for NAD(P)H oxidase. B: cells were exposed to 750 µM (each) 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) or other serine protease inhibitors with varying degrees of structural similarities to AEBSF for 15 min before treatment with 5-HT or vehicle. MAEBSF, 4-(2-n-methylamino)benzenesulfonyl fluoride; AEBSF, aminoethylbenzenesulfonyl fluoride; PMSF, phenylmethylsulfonyl fluoride; TLCK, Nα-p-tosyl-L-lysine chloromethylketone. Error bars, SE. *P < 0.5 vs. 5-HT value in presence of vehicle control. Reverse Bonferroni correction was used to correct for multiple comparisons.

Figure 4: Effect of NAD(P)H oxidase inhibitors on ERK phosphorylation. A: cells were exposed 100 µM 4′-hydroxy-3′-methoxyacetophenone (HMAP), 50 µM diphenyleneiodonium (DPI), 5 mM sodium azide, or 100 µM phenylarsine oxide (PAO) for 15–30 min before treatment with 5-HT or vehicle. Attenuation induced by PAO was reversed by coinoculation with British anti-Lewisite (BAL), as would be expected for NAD(P)H oxidase. B: cells were exposed to 750 µM (each) 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) or other serine protease inhibitors with varying degrees of structural similarities to AEBSF for 15 min before treatment with 5-HT or vehicle. MAEBSF, 4-(2-n-methylamino)benzenesulfonyl fluoride; AEBSF, aminoethylbenzenesulfonyl fluoride; PMSF, phenylmethylsulfonyl fluoride; TLCK, Nα-p-tosyl-L-lysine chloromethylketone. Error bars, SE. *P < 0.5 vs. 5-HT value in presence of vehicle control. Reverse Bonferroni correction was used to correct for multiple comparisons.
phosphorylation. In fact, PMSF was consistently shown to slightly increase ERK phosphorylation. Thus these studies strongly implicate an NAD(P)H oxidase as a major source of the ROS required to activate mesangial cell ERK.

We performed pathway mapping studies (Figs. 5–7), which showed that the ROS and ROS-generating enzyme are localized to a specific region of the signal transduction cascade initiated by 5-HT. Figure 5A places the ROS and NAD(P)H oxidase downstream of PKC, in that ERK phosphorylation resulting from direct activation of PKC by PMA can be attenuated by ROS scavengers (NAC and α-lipoic acid) and by two NAD(P)H oxidase inhibitors (DPI and AEBSF). Figure 5B shows that the ROS are downstream of PKC and NAD(P)H oxidase, in that inhibition of both enzymes fails to attenuate the activation of ERK by direct exposure of mesangial cells to H₂O₂. Figure 5C uses a similar strategy to probe the relative locations of MEK (which is one step upstream from ERK) to PKC and the ROS. Figure 5C demonstrates that the ROS scavenger NAC and the NAD(P)H oxidase inhibitor DPI block phosphorylation of MEK by PMA. Those studies place PKC and NAD(P)H oxidase upstream of MEK. Furthermore, when H₂O₂ was directly applied to mesangial cells, the phosphorylation of MEK was enhanced; this effect was attenuated by NAC, but not by DPI.

We assessed the intracellular production of H₂O₂ after administration of 5-HT. Figure 6 shows that application of 5-HT to quiescent, DCF-DA-loaded cells results in a time-dependent increase in fluorescence that was not present in vehicle-treated cells. These data demonstrate that 5-HT induces the rapid production of H₂O₂ in mesangial cells. Figure 6 shows that generation of H₂O₂ by 5-HT could be attenuated by DPI and NAC, confirming a role for NAD(P)H oxidase in the generation of H₂O₂ by 5-HT. Similar results (not shown) were obtained when PMA was used to increase production of H₂O₂ in mesangial cells, confirming a location for PKC that is upstream of the ROS. However, O₂⁻, and not H₂O₂, is the direct product of NAD(P)H oxidase. H₂O₂ could be produced from O₂⁻ by the action of SOD, but we believed that our contention that 5-HT induces production of ROS (H₂O₂ and O₂⁻) through NAD(P)H oxidase needed to be confirmed using an assay that measures the production of O₂⁻.

We tested the ability of 5-HT to increase the production of O₂⁻ by use of a cytochrome c reduction assay (Fig. 7). In their unstimulated state, rat mesangial cells produced ~200 pmol O₂⁻·min⁻¹·10⁶ cells⁻¹, and this was increased threefold after treatment with 1 µM 5-HT or 500 nM PMA. These effects were blocked by DPI and suppressed below baseline by inclusion in the assay mixture of SOD, which converts O₂⁻ to H₂O₂. They were also blocked by preincubation with GF-109203X, a specific inhibitor of PKC. Thus these studies are consistent with relative placements in the signaling cascade as follows: 5-HT₂A receptor → G protein → PLC → DAG → classical PKC → NAD(P)H oxidase → O₂⁻ → SOD → H₂O₂ → MEK → ERK.

DISCUSSION

Because the renal glomerulus could be exposed to 5-HT through local synthesis from the precursor molecule 5-hydroxytryptophan (36, 38) or by release from platelets or other infiltrating cells, 5-HT from several sources could modulate the function of glomerular resident cells. Rat renal mesangial cells express a 5-HT₂A receptor (28), the primary signaling pathway of which is thought to be activation of PLC (22, 40). However, in the glomerular mesangial cell, the signaling pathways linked to the 5-HT₂A receptor are quite diverse. They include phosphoinositide metabolism (40), liberation of Ca²⁺ derived from intracellular pools (27, 40), activation of PKC (40), stimulation of vasodilator prostaglandin synthesis (22), Cl⁻ conductance-related membrane depolarization, prolonged cytosolic alkalization related to activation of electroneutral Na⁺/H⁺

---

Fig. 5. Studies designed to establish relative locations of PKC, NAD(P)H oxidase, reactive oxygen species (ROS), mitogen-activated extracellular signal-regulated kinase (MEK), and ERK in signal transduction pathway. A and B: phospho-ERK blots obtained after cells were treated with inhibitors before treatment with 1 µM PMA, 1 mM H₂O₂, or vehicle for 15 min. C: phospho-MEK blots obtained after cells were treated with inhibitors for 15 min and then with 1 µM 5-HT, 1 µM PMA, 1 mM H₂O₂, or vehicle for 15 min. Error bars, SE. *P < 0.5 vs. 5-HT value in presence of vehicle control. Reverse Bonferroni correction was used to correct for multiple comparisons.
exchange, enhanced Na\textsuperscript{+}-independent Cl\textsuperscript{−}/HCO\textsubscript{3}{-} countertransport (27), inhibition of adenylyl cyclase (16), and activation of mitogenesis (40). The mitogenic response is of particular interest, in that it may play a key role in proliferative glomerulonephritis. Despite the diversity of signaling mechanisms available to the 5-HT\textsubscript{2A} receptor, little is known regarding the signal transduction pathways that mediate the mitogenic effect of 5-HT in mesangial cells.

The present studies support a role for PLC and classical forms of PKC (\(\alpha\), \(\beta\), or \(\gamma\)) in the stimulation of ERK initiated by the 5-HT\textsubscript{2A} receptor. That conclusion is based on the ability of overnight treatment of mesangial cells with phorbol esters or short-term treatment with PKC inhibitors to attenuate ERK phosphorylation. However, because those maneuvers are not specific for all types of PKC, we cannot comment on any potential roles for nonclassical PKC types in this process. Our results suggest that it is likely that classical forms of PKC mediate at least one-half of the stimulation by ERK, but they do not rule out an accessory role for other intermediates, such as nonclassical forms of PKC, or for tyrosine kinases.

Recent studies suggest that the ERKs are activated in response to mitogenic signals, hormones, cytokines, and growth factors in a variety of cell types (3). Excessive proliferation of resident glomerular cells can participate in the progression of chronic renal disease by altering glomerular architecture, synthesizing cytokines, or increasing the production of extracellular matrix in response to growth factors and other ligands. If unchecked, these processes can ultimately result in glomerular fibrosis. Oxidative stress and ROS production also could contribute to glomerular injury through several mechanisms. They may directly affect cellular function through lipid peroxidation of plasma membrane and subcellular membrane lipids and protein oxidation, thereby disrupting enzymatic functions. However, ROS are not necessarily always generated in quantities that are immediately cytotoxic and might function in some cases as second messengers (2). In the present study we have implicated H\textsubscript{2}O\textsubscript{2} and O\textsubscript{2}{\textsuperscript{−}} as major participants in the activation of ERK by 5-HT in mesangial cells.

5-HT is among many potential growth factors released from activated platelets. In models of glomerulonephritis, including chronic glomerulonephritis, mitogenic ligands (5-HT, thromboxanes, sphingolipids, phospholipids) released from platelets are thought to play a possible role in proliferation and/or fibrosis. Moreover, the potential importance of the ERKs in renal glomerular disease has been recently highlighted in an animal model of acute proliferative glomerulonephritis. Our data complement nicely those recently
reported by Bokemeyer et al. (6). They reported increased ERK activity in the renal cortex and glomeruli derived from an in vivo model of accelerated proliferative glomerulonephritis. Their studies suggested that macrophage activation resulted in increased ERK activation, since total body irradiation and resulting macrophage depletion reduced ERK activation in the model and protected the animals from progressive glomerulonephritis and proteinuria. Although they did not imply oxidative stress, it is possible that ROS generated and released from macrophages or cytokine-induced ROS generation by neighboring resident glomerular cells was in part responsible for ERK activation.

Wilmer et al. (43) recently implicated ROS as critical intermediates in the activation of ERKs in human mesangial cells by interleukin-1β. Interleukin-1β signals through a receptor that activates tyrosine phosphorylation reactions, although the receptor itself does not possess intrinsic tyrosine kinase activities. This receptor is of a class that does not typically signal directly through G proteins. Our results demonstrate that a receptor (5-HT₂A) that couples to cellular signaling cascades through Gq proteins also requires ROS as intermediate messengers for the activation of mesangial cell ERK. Moreover, this interaction fulfills five rigorous criteria implicating the ROS as second messengers in this system. First, antioxidants from two chemical classes attenuated the effects of 5-HT. Second, the effects of 5-HT were mimicked by direct application of three different oxidant molecules that interact with cellular thiols. Third, 5-HT was shown to produce measurable amounts of H₂O₂ and superoxide in a time scale similar to that of ERK activation. Fourth, a specific enzyme capable of generating ROS, NAD(P)H oxidase, was implicated by the use of four distinct types of inhibitors. Although NAD(P)H oxidase has classically been associated with neutrophils, two groups have used RT-PCR and immunoblot to document the presence of three or four subunits of the NAD(P)H oxidase in mesangial cells and glomerular podocytes (18, 21). Fifth, there was clear evidence of specificity in the actions of ROS on the ERK pathway. This last point is particularly important, in that ROS are short-lived, highly reactive molecules that are theoretically capable of eliciting some cellular effects through nonspecific toxicities. The effects on ERK are not likely to be nonspecific, because the ROS were localized to a specific region of the signal transduction pathway and because not every oxidant molecule applied to mesangial cells activated ERK. This second feature also has allowed us to generate the hypothesis that the ROS target a critical cysteine (rather than a methionine) residue.

Our studies are most consistent with an ERK activation pathway as follows: 5-HT₂A receptor → G protein → PLC → DAG → PKC → NAD(P)H oxidase → O₂⁻ → SOD → H₂O₂ → MEK → ERK. It is possible that MEK is the target of the ROS, although other signaling molecules could be targets as well. Ras and Raf are thought to be two steps and one step upstream, respectively, from MEK; although we did not specifically study their roles in this pathway, they are also likely potential targets for modification by ROS. It is also possible that an as yet unidentified protein could serve as the target in this transduction cascade.

Even though direct application of H₂O₂ results in ERK activation and 5-HT results in increased production of H₂O₂ in mesangial cells, other ROS might be involved. NAD(P)H oxidase does not directly produce H₂O₂, but O₂⁻ is probably converted to H₂O₂ by SOD. H₂O₂, in turn, could yield hydroxyl radicals through Fenton chemistry (15). Any of those free radicals could potentially serve as the second messenger that leads to ERK activation.

Our results correlate well with those recently described by Lee et al. (25), who showed that 5-HT-generated superoxide mediates ERK activation and thymidine incorporation in CCL-39 hamster lung fibroblasts and in bovine pulmonary artery smooth muscle cells. Our results differ in two respects: 1) the effect of 5-HT in those cell types is exclusively or predominantly mediated by 5-HT transporters, rather than by receptors; and 2) they focused on superoxide as a key mediator of the effects of 5-HT, whereas we studied H₂O₂. Our results should also be contrasted to those of Ushio-Fukai et al. (41), who recently showed that ANG II activates ERK in vascular smooth muscle cells through a pathway that is independent of NAD(P)H oxidase or H₂O₂. Thus NAD(P)H oxidase and/or H₂O₂ does not appear to be universally linked to ERK activation in all cells or by all G protein-coupled receptors.

In conclusion, these studies present evidence that a prototypical Gq-coupled receptor (5-HT₂A) can activate mesangial cell ERK through the generation of ROS. It is possible, although not yet proven, that this relationship is universal for all mesangial cell mitogens or mitogenic receptors. Because of the proposed roles of ERK and ROS in tissue damage and chronic renal disease processes, these studies underscore the need for a more detailed and mechanistic understanding of their interrelationships.

We thank Pamela Wackym for excellent technical assistance. This work was supported by the Department of Veterans Affairs (Merit Awards to J. R. Raymond and M. N. Garnovskaya), National Institutes of Health Grants DK-52448 and HL-03710 (to J. R. Raymond and E. L. Greene), a Robert Wood Johnson Faculty Development Award (to E. L. Greene), a laboratory endowment jointly supported by the Medical University of South Carolina Division of Nephrology and Dialysis Clinics, Inc. (to J. R. Raymond), an American Heart Association fellowship (to J. S. Grewal), and Medical University of South Carolina University Research Foundation awards (to M. N. Garnovskaya and E. L. Greene). M. N. Garnovskaya is a Research Scientist of the Department of Veterans Affairs.

Address for reprints and other correspondence: E. L. Greene, Rm. 829C CSB, Medical University of South Carolina, 171 Ashley Ave., Charleston, SC 29425 (E-mail: greeneel@musc.edu).

Received 27 August 1999; accepted in final form 3 November 1999.

REFERENCES

Downloaded from http://ajprenal.physiology.org/ by 102.20.33.6 on July 6, 2017


