The cellular redox state has been increasingly recognized as playing an important role in mediating cellular growth in response to growth factors and cytokines. We have previously reported that 5-hydroxytryptamine (5-HT) produces hyperplasia/hypertrophy of bovine pulmonary artery smooth muscle cells (BPASMCs) via its active transporter and signaling through tyrosine phosphorylation of GTPase-activating protein (GAP), Ras, and extracellular signal-regulated kinase (ERK)1/ERK2 mitogen-activated protein (MAP) kinase (7, 8, 24–26). O$_2^-$ released from vascular cells through activation of NAD(P)H oxidase is metabolized by superoxide dismutase (SOD) to H$_2$O$_2$ and then by catalase and glutathione peroxidase to form oxygen and water. The specific biological roles of O$_2^-$ and H$_2$O$_2$ are unclear, but recent evidence suggests that both of these by-products may serve in different systems as second messengers to activate multiple intracellular signaling pathways (14, 18, 36). Reactive oxygen species (ROS)-induced phosphorylation of ERK MAP kinase has been reported to occur in proliferation of vascular SMCs (9, 10, 13, 26, 28, 37, 39), fibroblasts (17), and renal mesangial cells (12). Recently, Greene et al. (11) reported that the 5-HT$_{2A}$ receptor mediates ERK MAP kinase activation via O$_2^-$ and H$_2$O$_2$ generation in rat mesangial cells. Our present study evaluates the role of intracellular H$_2$O$_2$ in the 5-HT-induced signal transduction pathway and cellular proliferation by inactivation of Cu/Zn SOD with copper-chelating agents and overexpression of catalase by infection with adenovirus. Both of these interventions inhibit 5-HT-induced cellular growth, and we conclude that O$_2^-$ serves as a source for H$_2$O$_2$ formation that is critical for the mitogenic response to 5-HT in BPASMCs.

In addition, we have explored the possible role of p38 MAP kinase in the proliferative responses of BPASMCs to 5-HT. Although this kinase has been previously shown to be responsive to angiotensin II and other stimuli (29, 31, 32, 40, 41), 5-HT failed to activate p38 MAP kinase in the BPASMCs, whereas SB-203580 and SB-202190, reported inhibitors of p38 MAP kinase, inhibited 5-HT-induced cellular growth. However, these inhibitors also inhibited 5-HT-induced O$_2^-$ release. Thus quenching of O$_2^-$ may be their mechanism for inhibition of cellular growth unrelated to p38 MAP kinase inhibition. These data indicate that generation of O$_2^-$ in BPASMCs in response to 5-HT is followed by an increase in intracellular H$_2$O$_2$ that mediates 5-HT-induced mitogenesis through activation of ERK1/ERK2 but not of p38 MAP kinase.

5-hydroxytryptamine; serotonin; reactive oxygen species; extracellular signal-regulated kinase; mitogen-activated protein kinase; mitogenesis

MATERIALS AND METHODS

Reagents. Phospho-specific p44/42 MAP kinase (Thr$^{202}$/Tyr$^{204}$) and phospho-specific p38 MAP kinase (Thr$^{180}$/Tyr$^{182}$) antibodies were from New England BioLabs (Beverly, MA). Anti-catalase antibody, SB-203580, and SB-202190 were...
from Calbiochem (San Diego, CA). WB-4101 HCl, (±)-8-hydroxy-dipropylaminoetrahydrobromide (DPAT), BW-723C86, α-methyl-5-hydroxytryptamine maleate (m-5-HT), R-(-)-2,5-dimethoxy-4-iodoamphetamine hydrochloride (DOI), clorgyline HCl, R-(-)-depenyl HCl, Ro-41-1049, and Ro-16-6491 were from Research Biochemicals International (Natick, MA). N,N’-diethyldithiocarbamate (Detc), catechol, tetraethylpentamine (TEPA), triethylenetetramine (TREN), and all other reagents were from Sigma Chemical (St. Louis, MO).

Cell culture. SMCs from bovine pulmonary artery were isolated and cultured by a modification of the method of Ross as previously described (24). Third- to fifth-passage SMCs were used.

Incorporation of [3H]thymidine. In brief, plated cells were cultured for 72 h in RPMI medium containing 10% FBS, followed by growth arrest in medium without FBS for 72 h. Cells were then incubated with and without 5-HT in the same medium for 20 h before being labeled with [methyl-3H]thymidine (0.1 mCi/ml, specific activity 20 Ci/mmol, New England Nuclear, Boston, MA) for 4 h. Four-hour preincubation with various inhibitory reagents was used to inhibit SOD and for buthionine sulfoximine (BSO) to inhibit biosynthesis of glutathione (2). Other inhibitors were added 30 min before the 5-HT. These agents alone, at the concentrations reported, did not alter the incorporation of [3H]thymidine into these cells. After labeling, experiments were terminated by aspiration of medium and washing of the cellular monolayer, first with ice-cold phosphate-buffered saline (PBS) and then with cold 6% trichloroacetic acid. Cells were then dissolved in 0.2 N NaOH, and radioactivity was counted.

Catalase infection. BPASMCs were infected with adenovirus containing catalase (AdSCMOCat) for 16 h using 20,000 and 40,000 particles/cell. Cells were growth arrested in medium without FBS for 72 h before exposure to 1 µM 5-HT and were then processed for [3H]thymidine incorporation or for electrophoresis. Infection with an adenovirus vector without the cDNA insert was done as a control. Adenovirus containing catalase infection was provided by Dr. John Englehardt, University of Iowa.

Measurement of O2− production in intact cells by a lucigenin-enhanced chemiluminescence assay. Cells were cultured in 100-mm petri dishes and growth arrested as described above. The assay was done as previously described (25). One micromolar 5-HT with and without other reagents was first added directly to the cellular monolayer. The cells were trypsinized, pelleted by centrifugation, and resuspended in PBS containing 10 mM glucose and 1 mg/ml bovine serum albumin. Cellular suspensions were loaded into a luminometer, and lucigenin (final concentration 500 µM) was automatically injected to start the reaction. A 15-s dark-adapted period was carried out before each sample reading in the luminometer. Photoemission was recorded with 60-s integration for 5–10 min with a Lumac Biocounter M2010 (Lumac System, Titusville, FL). Buffer blank, lucigenin, or other reagents used alone in these studies produced negligible chemiluminescence. Lucigenin recently has been reconfirmed to be a valid chemilumogenic probe for detecting O2− production by enzymatic and cellular systems compared with O2− consumption by oxygen polarography and a spin-trapping assay (27).

Preparation of whole cell extracts for electrophoresis. Cells were grown in 100-mm petri dishes and were growth arrested in medium as described above. The cells were preincubated with inhibitors for 1–2 h before the addition of 1 µM 5-HT for the periods indicated in RESULTS. Cellular monolayers were then washed twice with ice-cold PBS. Cell lysates were obtained by incubating the cellular monolayer in 1 ml of cell lysis buffer for 10 min at 4°C. The insoluble material was removed by centrifugation (14,000 g, 2 min), and the supernatant fraction was used for analysis. Twenty micrograms of protein of the whole cell lysate were subjected to SDS-PAGE on a Novex 12% precast gel (Novex, San Diego, CA).

RESULTS

We have previously reported that O2− is an important component of 5-HT signaling that produces mitogenesis (24–26). To investigate whether H2O2 formed by dismutation of O2− may be the actual signaling intermediate in the 5-HT-induced mitogenic process, we performed experiments both to inhibit Cu/Zn SOD with various inhibitory reagents and to reduce intracellular H2O2 by overexpression of catalase via adenovirus-modified gene transfer. We reasoned that both approaches would reduce the cellular stimulatory action of 5-HT if H2O2 were an intermediate in the action. First, we preincubated cells with DETC (0.4 and 0.8 mM), a copper-chelating agent, to inactivate Cu/Zn SOD (15). As anticipated, DETC further enhanced the 5-HT-induced intracellular O2− generation (Fig. 1) and dose-dependently inhibited the 5-HT-induced DNA synthesis of BPASMCs (Fig. 2A). Although less intense, similar inhibitions were observed when cells were pre- and coinubated with other copper-chelating reagents (20) such as catechol (1–10 µM), TRIEN (10–50 µM) and TEPA (5–50 µM), as shown in Fig. 2, B and C. These data suggest that dismutation of O2− to H2O2 is required for 5-HT-induced mitogenesis. 5-HT-induced [3H]thymidine incorporation by BPASMCs was also significantly inhibited in cells infected with adenovirus containing catalase cDNA (Fig. 3A). The adenoviral vector alone did not affect the stimulation of [3H]thymidine incorporation induced by 5-HT (Fig. 3A, control, 5-HT vs. control/vector, 5-HT/vector, respectively). Catalase is highly expressed in these infected BPASMCs (Fig. 3B, bottom). Inhibition of biosynthesis of glutathione by 10 µM BSO (2) did not affect 5-HT-induced DNA synthesis, suggesting that the cellular
redox state alone does not influence 5-HT-induced mitogenesis (data not shown).

5-HT activated ERK1/ERK2 MAP kinase three- to fourfold at 10 min of incubation (Fig. 3B, top), and this activation was inhibited when cells were infected with adenovirus containing catalase at 20,000 and 40,000 particles/cell (Fig. 3B, lanes 3 and 4). The adenoviral vector alone did not affect the 5-HT-induced activation of ERK MAP kinase (Fig. 3B, lane 5 vs. 2).

Although activation of ERK MAP kinase was induced by 5-HT, we failed to observe any activation of p38 MAP kinase (data not shown). Nevertheles, the p38 MAP kinase inhibitors SB-202190 and SB-203580 dose dependently inhibited 5-HT-induced [3H]thymidine incorporation, as shown in Fig. 4, A and B. These inhibitors were also found to block O₂ release from BPASMCs incubated with 5-HT (Fig. 5).

Various pathways for induction of cellular mitogenesis by 5-HT have been proposed via both the 5-HT transporter and 5-HT receptors (7). However, receptor agonists of 5-HT1A (1–10 μM of WB-4101 and DPAT), 5-HT2B (10 μM BW-723C86), and 5-HT2 (10 μM of m-5HT and DOI) all failed to show any enhancement in O₂ release or DNA synthesis in BPASMCs (data not shown).

Exogenous application of H₂O₂ to BPASMCs activated ERK1/ERK2 MAP kinase dose and time dependently as shown in Fig. 6A but failed to induce DNA synthesis in this cell type (Fig. 6B).

DISCUSSION

O₂ and H₂O₂ are now well-recognized intermediates in cell signaling for a large variety of ligands (38). We have previously demonstrated that O₂ participates in the signaling pathway by which 5-HT induces cellular mitogenesis (25) and have related this action to the 5-HT transporter as opposed to a 5-HT receptor for certain cell types (7). Stimulation of the transporter by 5-HT activates p21 Ras through tyrosine phosphorylation, leading to formation of O₂ that, in turn, activates ERK1/ERK2 MAP kinases. In association with other steps in a signaling cascade, cellular mitogenesis en-

![Fig. 1. Preincubation of bovine pulmonary artery smooth muscle cells (BPASMCs) with 0.8 mM N,N'-diethyldithiocarbamate (DETC) further enhanced superoxide (O₂) generation induced by 1 μM 5-hydroxytryptamine (5-HT) alone. Experiments were done as noted in MATERIALS AND METHODS. Values are means ± SD; n = 4. Significantly different (P < 0.05) from: * quiescent control (Contl); ** 5-HT.

![Fig. 2. Effects of inhibition of copper-zinc superoxide dismutase (Cu/Zn SOD) (DETC (A), catechol and triethylenetetramine (TRIEN; C)) on 5-HT-induced [3H]thymidine ([3H]-TdR) incorporation. BPASMCs were exposed to 5-HT (1 μM) in the presence or absence of inhibitors for 24 h. Preincubation for 4 h with inhibitors was used. cpm, Counts/min. Values are means ± SD; n = 4: Significantly different (P < 0.05) from: * quiescent control; ** 5-HT.

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The present experiments were undertaken to determine whether dismutation of $\text{O}_2$ to form $\text{H}_2\text{O}_2$ might be a critical step in the signaling process. As anticipated, inhibition of Cu/Zn SOD by treatment with DETC further elevated $\text{O}_2$ in cells treated with 5-HT. Stimulation of 5-HT-induced cellular mitogenesis was blocked by inhibition of SOD and was prevented by infection of cells with catalase, thereby supporting the concept that $\text{H}_2\text{O}_2$ is important in the signaling process. A variable extent of inhibition by the copper chelators was noted, but all produced the same qualitative effect.

ERK MAP kinase activation is now well recognized to be associated with cellular proliferation induced by growth factors and cytokines through the formation of ROS. Aside from the studies with 5-HT, angiotensin II is the most widely examined vasoactive agent that signals through ROS and MAP kinase. However, activation of specific pathways for angiotensin II-induced SMC hyperplasia/hypertrophy remains controversial.

Some laboratories have reported that ROS participate in angiotensin II-induced protein tyrosine phosphorylation of receptor tyrosine kinases and mediate the downstream signaling events including activation of ERK MAP kinase (1, 9, 34, 43, 44). Others (40, 41) have reported that c-Jun amino-terminal kinase (JNK) and p38 MAP kinase, but not ERK, are the critical redox-sensitive signaling pathways activated by angiotensin II. p38 MAP kinase is a Ser/Thr protein kinase activated by various inflammatory cytokines and a variety of stress stimuli (29, 31, 34, 35), but it also acts as a negative regulator of cell proliferation (3, 22). Our studies have shown that p38 MAP kinase activation is not in the intermediate signaling pathway for 5-HT (data not shown); rather, the participation of SB-202190 and SB-203580 (inhibitors of p38 MAP kinase) in the blockade of 5-HT-induced DNA synthesis is more likely through $\text{O}_2$ quenching or inhibition of NAD(P)/H oxidase or another unknown target, as reported by Lal et al. (21) and Hunt et al. (16).

Recently, production of ROS ($\text{O}_2$ and $\text{H}_2\text{O}_2$) through a 5-HT$_2$A receptor (11) and via transfection with a...
human 5-HT1A receptor (30) was reported. The conclusion of Greene et al. (11) that the 5HT2A receptor is involved in the mitogenic process is dependent on data showing that ketanserin, as a 5-HT2A antagonist, inhibits the mitogenesis induced by 5-HT in rat mesangial cells. However, ketanserin has also been reported to inhibit 5-HT uptake, and the serotonin transporter has been recently identified in this cell type (33). We have also observed active 5-HT transport in the Chinese hamster ovary (CHO) fibroblast of both wild-type and 5-HT1A-transfected cells (Lee SL, Raymond JR, Wang WW, and Fanburg BL, unpublished data). Mutant mice lacking the serotonin transporter are protected from developing pulmonary hypertension (5). These observations support a previous contention (4–6, 24) that 5-HT signals mitogenesis in SMCs through a 5-HT transporter. We also have previously reported that, unlike BPASMCs, where the 5-HT transporter predominates in 5-HT-induced cellular mitogenesis, CHO lung fibroblasts signal via both a 5HT2 receptor and a 5-HT transporter (26). Agonists of 5-HT receptors 1A, 2B, and 2, however, failed to induce O22 and H2O2 generation and activation of ERK MAP kinase in BPASMCs, as has also been reported for mesangial cells and fibroblasts (11, 30). These data suggest that 5-HT-induced mitogenic signaling in BPASMCs is mediated through the 5-HT2 receptor but not through the 5-HT1A (23) or 5-HT2 receptor or absence of the 5-HT2 receptor in this cell type. Whether a 5-HT receptor or transporter or both participate in mitogenic signaling varies with the cells used and needs to be carefully characterized for each cell type (8).

Recently Jin et al. (19) reported that exogenously added H2O2-induced egr-1, fra-1 and c-jun gene expression is mediated through platelet-derived growth factor receptor tyrosine kinase activation. Our data show that the extracellular application of 0.01–50 μM H2O2 activated ERK MAP kinase but failed to induce mitogenesis. This is consistent with observations by Rao (35), who found that H2O2 induced eukaryotic translation initiation factor 4E phosphorylation and expression of c-fos, c-jun, and Hsp70 mRNA but failed to stimulate hypertrophy in SMCs. Because of this observation, Rao questioned the role of intracellular H2O2 in angiotensin II-induced hypertrophy as proposed by Zafar et al. (44) and concluded that these activations were associated with cell survival. Application of H2O2 extracellularly may differ considerably in cellular response from that generated intracellularly at specific locations in response to an applied signaling agent such as 5-HT or angiotensin II.

The mitochondrial enzyme monoamine oxidase B has been recently reported to generate H2O2, which may lead to ERK-dependent cellular mitogenesis (42). This enzyme is unlikely to be involved in 5-HT-induced mitogenesis because we failed to observe any inhibition by monoamine oxidase type A (10 μM of clorgyline and Ro-41-10490) or type B (10 μM of deprenyl and Ro-16-6491) inhibitors (data not shown). Our previous data showing that O22 is required for 5-HT-induced mitogenesis (25) and the present data showing that overexpression of catalase by infection with adenovirus containing catalase inhibits 5-HT-induced ERK MAP kinase activation and cellular proliferation strongly suggest that intracellularly released O22 serves as a source for H2O2 generation that then mediates the mitogenic response to 5-HT in BPASMCs. The specific oxidase responsible for the generation of O22 and H2O2 in the case of 5-HT is presently unknown. Furthermore, until better information is available about the enzyme kinetics, microenvironment, and physical chemistry of the target for oxidant signaling, it will be difficult to ex-
plain why some pathways seem to utilize O$_2$ and others H$_2$O$_2$ for cell signaling.

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