5-Hydroxytryptamine$_{1A}$ receptor/G$_i\beta\gamma$ stimulates mitogen-activated protein kinase via NAD(P)H oxidase and reactive oxygen species upstream of Src in Chinese hamster ovary fibroblasts

Yurii V. MUKHIN*†, Maria N. GARNOVSKAYA*†, Georgiann COLLINSWORTH*†, Jasjit S. GREWAL*†, DeKisha PENDERGRASS*†, Toshio NAGAI*†, Stephen PINCKNEY*†, Eddie L. GREENE*†, and John R. RAYMOND*†[1]

*Department of Medicine, Medical University of South Carolina, 96 Jonathan Lucas Street, Charleston, SC 29425, U.S.A., and
†Ralph H. Johnson Veterans' Affairs Medical Center, 109 Bee Street, Charleston, SC, 29401, U.S.A.

The hypothesis of this work is that the 'serotonin' or 5-hydroxytryptamine (5-HT)$_{1A}$ receptor, which activates the extracellular signal-regulated kinase (ERK) through a G$_i\beta\gamma$-mediated pathway, does so through the intermediate actions of reactive oxygen species (ROS). Five criteria were shown to support a key role for ROS in the activation of ERK by the 5-HT$_{1A}$ receptor. (1) Antioxidants inhibit activation of ERK by 5-HT. (2) Application of cysteine-reactive oxidant molecules activates ERK. (3) The 5-HT$_{1A}$ receptor alters cellular redox properties, and generates both superoxide and hydrogen peroxide. (4) A specific ROS-producing enzyme [NAD(P)H oxidase] is involved in the activation of ERK. (5) There is specificity both in the effects of various chemical oxidizers, and in the putative location of the ROS in the ERK activation pathway. We propose that NAD(P)H oxidase is located in the ERK activation pathway stimulated by the transfected 5-HT$_{1A}$ receptor in Chinese hamster ovary (CHO) cells downstream of G$_i\beta\gamma$ subunits and upstream of or at the level of the non-receptor tyrosine kinase, Src. Moreover, these experiments provide confirmation that the transfected human 5-HT$_{1A}$ receptor induces the production of ROS (superoxide and hydrogen peroxide) in CHO cells, and support the possibility that an NAD(P)H oxidase-like enzyme might be involved in the 5-HT-mediated generation of both superoxide and hydrogen peroxide.

Key words: G protein, phosphorylation, proliferation, serotonin receptor, tyrosine kinase.

INTRODUCTION

Reactive oxygen species (ROS), such as superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^\bullet$), were originally characterized in leukocytes as critical intermediaries in the cellular defence mechanism against infectious agents. It is logical that cells should be able to generate toxic substances to ward off threats from micro-organisms, and that the production of those toxic substances would be tightly regulated. It might appear less logical that cells would actually use ROS as second messengers. Nevertheless, there is mounting evidence for roles of various ROS as second messengers, particularly for $O_2^{-\bullet}$ and H_2O_2. For example, in neutrophils, PC12 phaeochromocytoma cells and vascular smooth muscle cells (VSMC), oxidants activate extracellular signal-regulated protein kinase (ERK) and mitogen-activated protein kinases (MAPKs) through the tyrosine phosphorylation of mitogen and ERK kinase (MEK) and inhibition of ERK phosphatases [1,2]. They also stimulate growth and proto-oncogene expression [3,4], and stimulate thymidine incorporation (marker of DNA synthesis) [3].

Various types of receptors have been shown to induce the production of ROS in cells, and, in some cases, the ROS have been linked to the activation of MAPKs. The best examples for involvement of ROS in the activation of the ERK are provided by tyrosine kinase-linked growth factor receptors such as the interleukin 1 [5], platelet-derived growth factor [6], and epidermal growth factor ('EGF') receptors [6]. Until recently, roles for G-protein-coupled receptors have been less well explored. The lysophosphatidic acid receptor requires ROS and transactivation of the epidermal growth factor receptor to stimulate ERK in HeLa cells [7,8]. In VSMC, however, angiotensin II-generated ROS stimulate p38 MAPK and not ERK [9]. Thus, the sensitivity of ERK to oxidative signals is controversial and appears to vary from cell type to cell type [3,10–14], and there is no consensus on the location(s) of the ROS vis-à-vis the known components of the ERK activation pathways.

There are two major pathways through which G-protein-coupled receptors can activate ERK, and there is evidence for the role of ROS in both pathways. The first pathway involves activation of protein kinase C (PKC), usually through the intermediate actions of G-protein α-subunits and phospholipases [15]. Subsequent to PKC activation, Raf-1 (a MAPK kinase kinase) is phosphorylated and activated, and in turn, it sequentially activates MEK and ERK. In this pathway, there is approximately equal evidence for a location of the ROS upstream [16] and downstream [17] of PKC. The second pathway involves

Abbreviations used: AEBSF, 4-(2-aminoethyl)-benzenesulphonyl fluoride; AEBSAC, 4-(2-aminoethyl)-benzenesulphonic acid; AEBSNH$_2$, 4-(2-aminoethyl)-benzenesulphonamide; α-LA, α-lipoic acid; BAL, British anti-Lewisite, 2,3-dimercaptopropanol; BSO, buthionine sulphoximine; CHO, Chinese hamster ovary; DCF-DA, 2′,7′-dichlorofluorescin diacetate; diamide, azodicarboxylic acid bis(dimethylamide); DPI, diphenylene iodinium; 5-HT, 5-hydroxytryptamine or serotonin; ERK, extracellular signal-regulated kinase; HMAP, 4′-hydroxy-3′-methoxyacetophenone; MAEBSF, 4-(2-*N*-methylaminoethyl)-benzenesulphonyl fluoride; MAPK, mitogen-activated protein kinase; MEK, mitogen and extracellular signal-regulated kinase kinase; NAC, *N*-acetylcysteine; PAO, phenylarsine oxide; PKC, protein kinase(s) C, calcium and phospholipid-dependent protein kinases; PP1, 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo(3,4-*d*)pyrimidine; ROS, reactive oxygen species; SOD, superoxide dismutase; TBH, *tert*-butylhydroperoxide; TLCK; *N*α-*p*-tosyl-L-lysine chloromethylketone; VSMC, vascular smooth muscle cells.

[1] To whom correspondence should be addressed (e-mail raymondj@musc.edu).

activation of Src (a tyrosine kinase), followed by phosphorylation of Shc (a docking platform), and recruitment of an adapter protein (Grb2) and the Ras-specific G protein activator, Sos. This results in the activation of the small G protein, Ras, and in the recruitment of Raf kinase to the plasma membrane, where it becomes activated. Similar to the PKC pathway, there is no consensus on the location of ROS in this pathway. There is some evidence that ROS may work upstream of MEK [12,13,18], but other studies place ROS downstream of MEK [19,20].

The goals of the current study were to determine whether there is involvement of ROS in the activation of ERK in the G$\beta\gamma$/Src/Shc/Grb2/Sos/Ras/Raf/MEK pathway, and to map the location of the ROS vis-à-vis the known components of this pathway. To accomplish those goals, we used an expression system in which the activation of ERK has been characterized as occurring exclusively through the G$\beta\gamma$ pathway [21,22]. This system consists of Chinese hamster ovary (CHO) cells, which have been transfected to stably express the G_i-coupled 5-hydroxytryptamine $(5\text{-HT})_{1A}$ ('serotonin') receptor [23]. The use of this system reduced potential ambiguities that might arise when studying a more heterogeneous population of receptors.

EXPERIMENTAL

Materials

Reagents were obtained from the following sources: 4-(2-aminoethyl)-benzenesulphonyl fluoride (AEBSF), arsenite, ATP, BSA, British anti-Lewisite (BAL) or 2,3-dimercapto-propanol, buthionine sulphoximine (BSO), cytochrome *c*, 2′,7′-dichlorofluorescin diacetate (DCF-DA), azodicarboxylic acid bis(dimethylamide)(diamide), diphenylene iodinium (DPI), 5-HT, hydrogen peroxide, α-LA (α-lipoic acid) (reduced form), 4-(2-*N*-methylaminoethyl)-benzenesulphonyl fluoride (MAEBSF), menadione, *N*-acetylcysteine (NAC), pertussis toxin, phenylarsine oxide (PAO), potassium ferricyanide, potassium ferrocyanide, sodium azide, and *N*α-*p*-tosyl-L-lysine chloromethylketone (TLCK) were from Sigma (St. Louis, MO, U.S.A.). 4-(2-Aminoethyl)-benzenesulphonamide ($AEBSNH_2$), 4′-hydroxy-3′-methoxyacetophenone (HMAP), and *tert*-butylhydroperoxide (TBH) were from Aldrich (Milwaukee, WI, U.S.A.). PD98059 and 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo-(3,4-*d*)pyrimidine (PP1) were from Calbiochem (La Jolla, CA, U.S.A.). Cell culture media, serum and antibiotics were obtained from Gibco BRL (Gaithersburg, MD, U.S.A.), and culture flasks from Costar (Cambridge, MA, U.S.A.). The phospho-ERK and phospho-MEK kits were obtained from New England Biolabs (Beverly, MA, U.S.A.). The Vistra™ system for enhanced luminescent detection was purchased from Amersham (Sunnyvale, CA, U.S.A.).

Phospho-ERK immunoblots

ERK phosphorylation was assessed using a phosphorylation-state-specific ERK antibody (New England Biolabs, Beverly, MA, U.S.A.) as previously described [23].

Fluorescent measurement of intracellular oxidant generation

The H_2O_2-sensitive fluorescent probe, DCF-DA was used to assess the generation of intracellular ROS [9,24]. Cells in monolayer were incubated with Earle's balanced salt solution supplemented with 10 μM DCF-DA and 1 % (w/v) BSA for 30 min at 37 °C. The supernatant was removed and replaced with fresh unsupplemented Earle's solution prior to stimulation with 5-HT, which was added from a 1000-fold concentrated stock solution directly to the Earle's solution prior to analysis. Relative fluorescence intensity and fluorescent images were obtained over time (0.5–20 min) using laser confocal scanning microscopy (LSMGB-200, Olympus Optical Co., Ltd., Tokyo, Japan) at an excitation wavelength of 485 nm; emission was measured at a wavelength of 530 nm.

Measurement of superoxide anion production

$O_2^{-\bullet}$ production was quantified by the cytochrome *c* reduction assay [25] with modifications. In brief, 4×10^6 cells were grown in 100 mm plates and starved for 12 h in serum and Phenol Red-free RPMI 1640 medium. Cells were then washed twice with PBS, and were incubated with 200 μM cytochrome *c*. Cells were incubated with vehicle or 1 μM 5-HT, with or without inhibitors for 60 min. Cells were pretreated with inhibitors for 30 min before applying 5-HT. Absorbance of 1 ml of cell-free supernatant was measured at 550 nm. Blanks contained 300 units of superoxide dismutase (SOD) in addition to 200 μM cytochrome *c*. The following equation was used to determine $O_2^{-\bullet}$ produced in nmoles:

$$\text{nmol of } O_2^{-\bullet}/10^6 \text{ cells} = [A_{550}(\text{without SOD}) - A_{550}(\text{in the presence of SOD})] \times 159$$

Potentiometric measurement of cellular reduction rate

We used a Cytosensor™ microphysiometer with custom-modified gold electrodes (Molecular Devices, Sunnyvale, CA, U.S.A.) and an extracellular solution containing ferricyanide/ferrocyanide to assess the redox state of CHO cells by an electrochemical potentiometric method. Menadione/menadiol was added to the perfusate as a shuttle for carrying electrons across the plasma membrane. This procedure has been validated for several cell types, including CHO cells, and described in detail recently [26]. Cells (2.5×10^5) were seeded on polycarbonate inserts (Costar, Cambridge, MA, U.S.A.) the night prior to experimentation. After attachment, they were incubated in serum-free Ham's F12 medium supplemented with 0.5 % BSA and antibiotics until the following day, when they were placed into microphysiometer chambers. Prior to each experiment, the chambers and electrodes were washed with distilled water and equilibrated with redox medium consisting of phosphate buffered saline (145 mM Na^+, 4 mM K^+, 1 mM Mg^{2+}, 1 mM Ca^{2+}, 147 mM Cl^-, 10 mM phosphate, pH 7.4) supplemented with 10 mM glucose, 20 mM Hepes, 1 mg/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. After calibration to zero volts during perfusion in the absence of menadione/menadiol, measurements were made by switching to redox media containing 10 μM menadione. Cells were perfused with this medium, then switched to identical medium (to control for valve switching artefacts) or medium containing test compounds. The chambers were perfused for 60 s, then flow was stopped for 30 s during each 90 s pump cycle. The rate of change in the potential was measured during seconds 18–28 of the stop flow period. The long delay between stopping the perfusion and measuring the reduction rate was needed, because the gold electrodes do not equilibrate with the extracellular ferricyanide/ferrocyanide redox pair as rapidly as the standard pH-sensing electrodes equilibrate with protons [26]. 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.

Transfection of CHO cells

The clone expressing the human 5-HT$_{1A}$ receptor has been previously described [22,23]. Transient expression of the other cDNA constructs was achieved by transfection in the presence of 10–15 μl of lipofectAMINE™ (2 h in serum- and antibiotic-free medium). Two days prior to assays, cells were transfected with 1 μg of the various plasmids. Transfection efficiency (> 90 %) was evaluated by co-transfecting with 0.2 μg of cDNA (with the sequence modified to account for human codon preference) encoding the green fluorescent protein from the jellyfish *Aequorea victoria* (Clontech, Palo Alto, CA, U.S.A.) as a marker. Original sources of the other cDNA and the literature citations are described in detail in reference [22].

RESULTS

Antioxidant compounds block activation of ERK

Three structurally distinct antioxidants, ascorbic acid, α-LA (reduced form) and NAC [27,28], each slightly suppressed the basal level of ERK phosphorylation, and markedly inhibited the stimulation of ERK by 5-HT (Figure 1A). The effect of α-LA could be reversed by co-incubation with 400 μM of racemic BSO, an inhibitor of γ-glutamylcysteine synthetase, which is the rate-limiting enzyme in glutathione synthesis. Because BSO treatment should deplete cells of the major endogenous antioxidant, glutathione [27,28], these results are consistent with a role for oxidant molecules in the activation of ERK.

Direct application of oxidant molecules activates ERK

We applied four different oxidative compounds (H_2O_2, TBH, diamide, and sodium arsenite) directly to CHO cells, and examined the phosphorylation of ERK. Each is known to interact with cellular thiols, particularly with cysteine and methionine

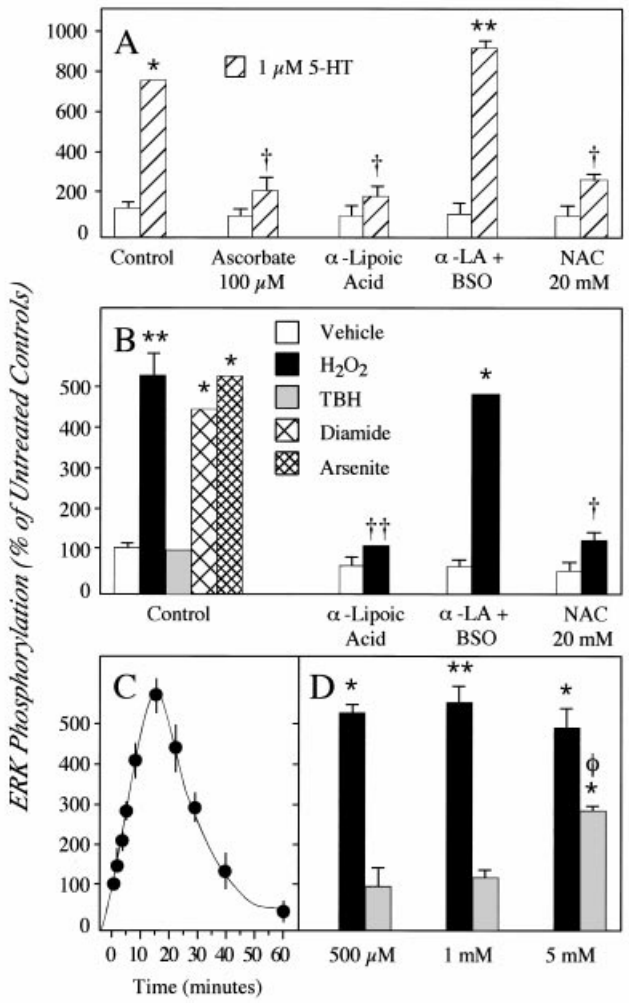

Figure 1 Antioxidant compounds block activation of ERK

(**A**) Cells were pre-incubated with redox-regulating compounds for 30 min (NAC, ascorbate) or overnight (BSO, α-LA), prior to treatment with 1 μM 5-HT or without (open bars) for 10 min. Cells were then rapidly lysed, and the extracts were analysed for phosphorylated ERK by immunoblot. (**B**) Effects of various redox regulating compounds on the phosphorylation of ERK. Cells were incubated for 10 min with various oxidants (H_2O_2, TBH, diamide or arsenite) after preincubation with α-LA, BSO or NAC (20 mM). (**C**) Time course of ERK phosphorylation induced by 500 μM H_2O_2. (**D**) Comparison of ERK phosphorylation induced by H_2O_2 (black bars) and TBH (grey bars). Experiments were performed in triplicate at least three times each. Paired one-tailed *t* tests were used for statistical analyses, and reverse Bonferroni was used to correct for multiple comparisons. $^*P < 0.05$ compared with baseline; $^{**}P < 0.01$ compared with the baseline; †$P < 0.05$ compared with the 5-HT-stimulated value; ††$P < 0.01$ compared with 5-HT-stimulated value; ϕ $P < 0.05$ when H_2O_2 is compared with TBH.

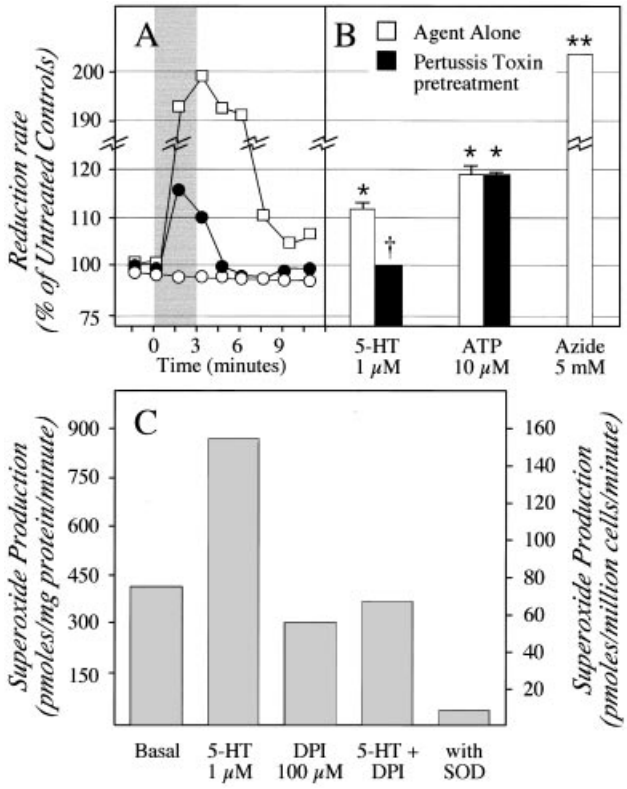

Figure 2 Evidence that 5-HT induces generation of ROS in CHO cells

(**A**) Measurement of cellular reduction rate using a microphysiometer and a menadione/menadiol ferricyanide/ferrocyanide shuttle system as described in the Experimental section. The grey background represents the period of 5-HT treatment. Representative plots are shown from cells treated with 1 μM 5-HT (●), chamber containing 5-HT, but no cells (○), or cells treated with 250 μM diamide (□). (**B**) Stimulation of the reduction rate in CHO cells by 5-HT, ATP, and sodium azide in cells pretreated with (filled bars) or without (open bars) pertussis toxin (200 ng/ml for 18 h). (**C**) Measurement of superoxide production by the cytochrome *c* reduction assay. Paired one-tailed *t* tests were used for statistical analyses, and reverse Bonferroni was used to correct for multiple comparisons. $^*P < 0.05$ compared to baseline; $^{**}P < 0.01$ compared to baseline; †$P < 0.05$ compared to the 5-HT-stimulated value.

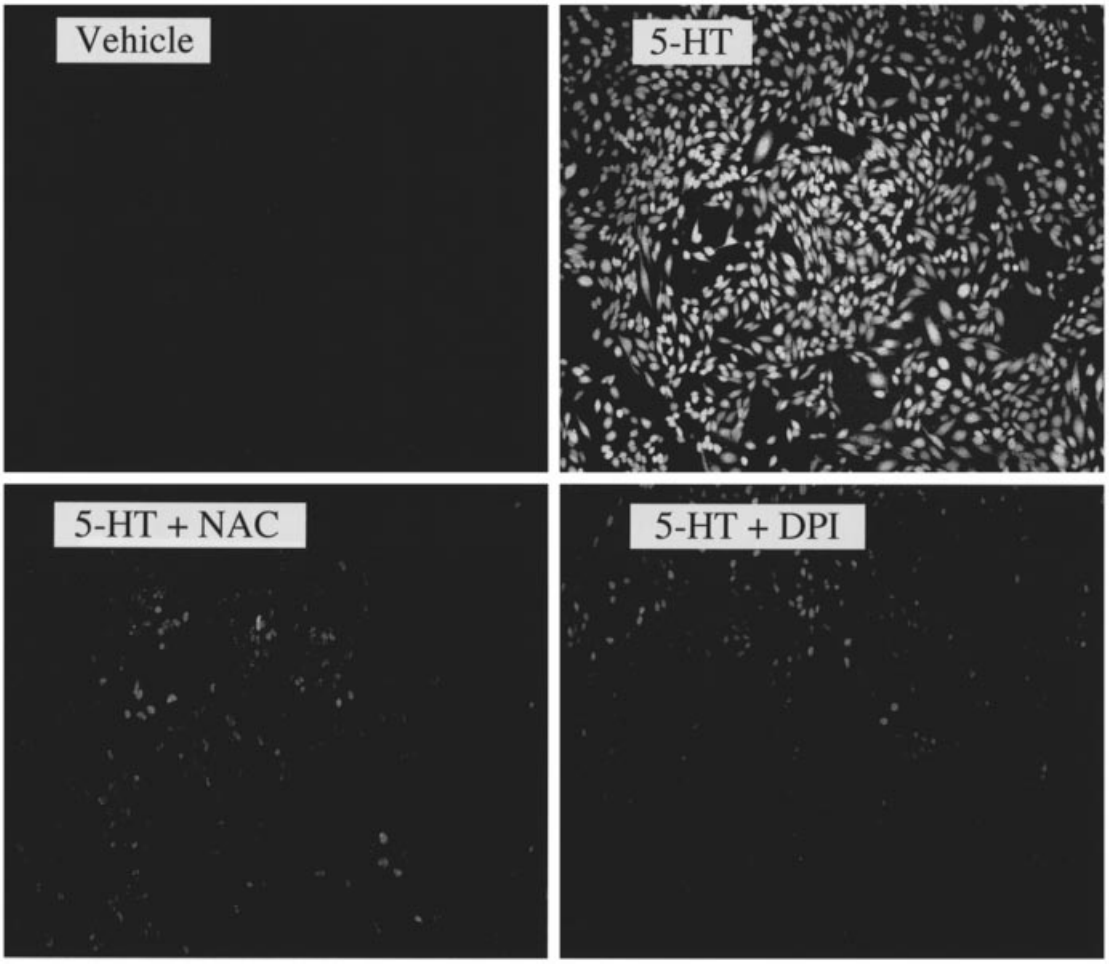

Figure 3 Fluorescent indicator evidence that 5-HT induces generation of H_2O_2 in CHO cells

Cells were loaded with DCF-DA as described in the Experimental section. After an additional incubation for 30 min with vehicle, 20 mM NAC or 50 mM DPI, cells were treated with vehicle or 1 μM 5-HT for 30 min. The interaction of internally generated H_2O_2 with the DCF-DA causes individual cells to have a brighter fluorescence (magnification 16 $\times$). These experiments were repeated at least three times for each agent.

residues [27]. However, because of its bulky butyl group, TBH is relatively selective for methionine [28]. Application of 400 μM diamide, 500 μM H_2O_2, and 500 μM sodium arsenite induced a rapid phosphorylation of ERK, whereas 500 μM TBH did not (Figure 1B). The stimulatory effect of H_2O_2 was markedly diminished in cells pretreated briefly with NAC or overnight with α-LA, but was not diminished in cells pretreated with both BSO and α-LA. The peak effect for 500 μM H_2O_2 occurred at 10–15 min, and returned to baseline within one h (Figure 1C). Further, we compared the ability of several concentrations of H_2O_2 and TBH to induce ERK phosphorylation. Despite having similar oxidative capacity, the two compounds exhibited markedly different abilities to phosphorylate ERK (Figure 1D). Thus oxidant-generating chemicals mimic the effect of 5-HT on ERK and this effect is specific for certain oxidant compounds.

Application of 5-HT to CHO cells leads to production of ROS

Three different methods were used to document alterations of cellular redox potential induced by 5-HT. We first used a Cytosensor™ microphysiometer that was retro-fitted with redox sensors to measure cellular reducing power. Figure 2(A) shows representative tracings of cell monolayers exposed for 3 min to 1 μM 5-HT (grey background) in the presence (●) or the absence of cells (○). In chambers with cells, 5-HT increased the reduction rate by $12 \pm 2\%$ ($n = 5$, $P < 0.05$). 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. Similar results were obtained by adding 250 μM diamide, a powerful oxidizing agent, to the medium (□). Diamide ($n = 3$) induced an $\approx 110\%$ increase in the reduction rate, confirming that alterations of cellular redox potential can be detected by the modified sensors. Figure 2(B) shows the peak effect of 1 μM 5-HT with (filled bars) and without (open bars) prior pertussis toxin treatment (200 ng/ml for 18 h). Pertussis toxin treatment abolished the ability of 5-HT to change the cellular reduction rate. The effect of pertussis toxin is not due to non-specific cellular toxicity, because 10 μM ATP, which couples through non-pertussis toxin-sensitive G proteins, increased reduction rate equally with or without preincubation with pertussis toxin ($18 \pm 3\%$ and $18 \pm 1\%$, respectively; $n = 3$). Blockade of the mitochondrial electron transport chain has been previously shown to markedly increase cellular production of H_2O_2 [29], and to increase the reduction rate in CHO cells [26]. Perfusion of

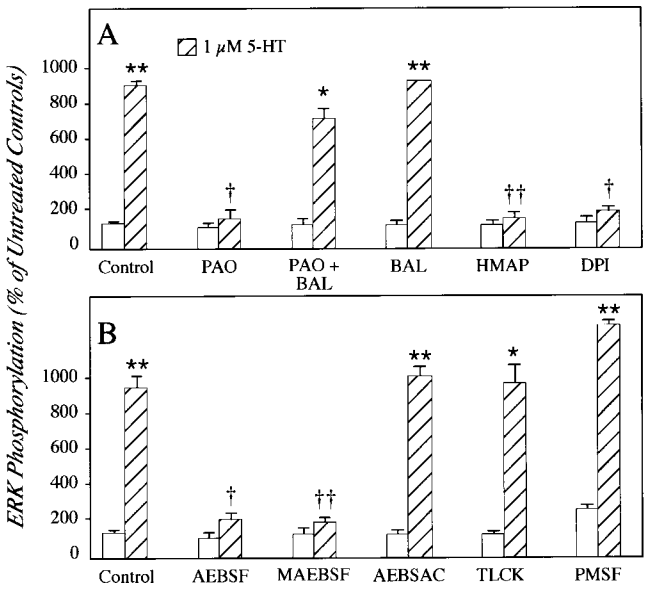

Figure 4 Inhibitors of NAD(P)H oxidase block phosphorylation of ERK induced by 5-HT

(**A**) Cells were preincubated with PAO, HMAP or DPI for 30 min prior to treatment with (hatched bars) or without (open bars) 1 μM 5-HT. Cells treated with 20 μM BAL were incubated for an additional 30 min prior to the addition of PAO or vehicle. (**B**) Cells were preincubated with a series of protease inhibitors for 30 min prior to stimulation with (hatched bars) or without (open bars) 5-HT. MAEBSF and AEBSF blocked ERK phosphorylation, but AEBSAC, PMSF, and TLCK did not. Each inhibitor was used at 750 μM. Paired one-tailed *t* tests were used for statistical analyses, and reverse Bonferroni was used to correct for multiple comparisons. $^*P < 0.05$ compared to baseline; $^{**}P < 0.01$ compared to baseline; †$P < 0.05$ compared to the 5-HT-stimulated value; ††$P < 0.01$ when compared with the 5-HT-stimulated value.

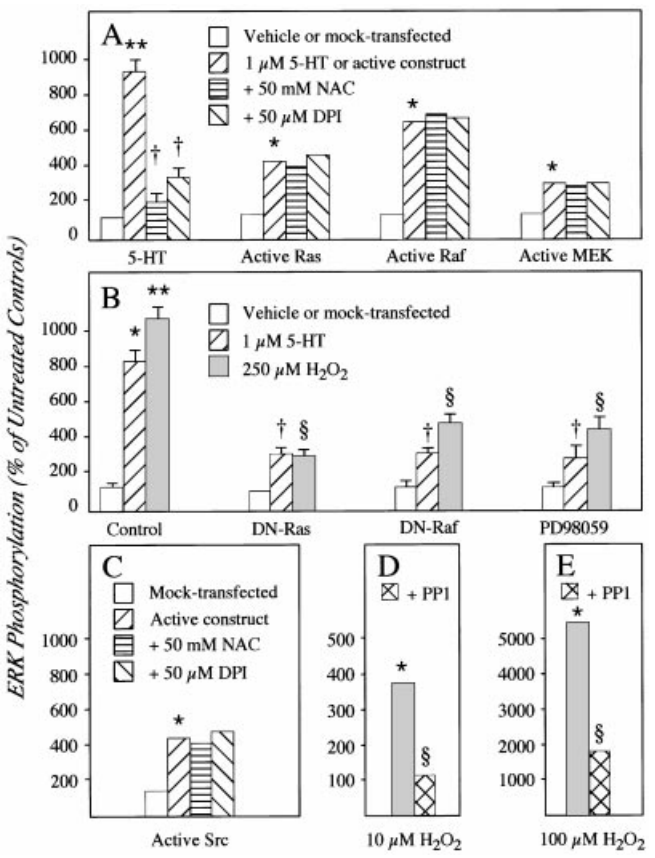

Figure 5 Mapping studies

(**A**) Effects of NAC and DPI on ERK phosphorylation induced by 5-HT or transfection with cDNA constructs encoding activated Ras, activated Raf, and activated MEK. An empty plasmid (pCDNA3) was used for mock transfection controls. Cells were studied 36–48 h after transfection. Cells were incubated for up to 2 h with inhibitors without effects on ERK phosphorylation. (**B**) Effects of dominant negative (DN) Ras and Raf constructs and 20 μM PD98059 (MEK inhibitor) on ERK phosphorylation induced by 5-HT or 250 μM H_2O_2. For the experiments using dominant negative Ras and Raf constructs, cells were studied 36–48 h after transfection. Cells were treated with vehicle, 5-HT or H_2O_2 for 10 min, then lysed for analyses of phospho-ERK content. (**C**) Lack of effect of NAC and DPI on ERK phosphorylation induced by transfection with constitutively activated Src. (**D**) and (**E**) Cells were incubated for 1 h with 10 μM PP1 (Src inhibitor) prior to exposure to 10 or 100 μM H_2O_2 for 10 min (cross-hatched bars). No prior incubation with PP1 (grey bars). ERK phosphorylation was assessed by immunoblot as described in the Experimental section. Paired one-tailed *t* tests were used for statistical analyses, and reverse Bonferroni was used to correct for multiple comparisons. $^*P < 0.05$ compared to baseline; $^{**}P < 0.01$ compared to baseline; †$P < 0.05$ compared to the 5-HT-stimulated value in control cells; §$P < 0.05$ compared to the H_2O_2-stimulated value in control cells. Experiments were performed at least three times, except for those with PD98059, which were performed twice.

cells with sodium azide (which blocks the mitochondrial electron transport chain at site IV) resulted in a rapid and pronounced increase in reduction rate ($103 \pm 15\%$, $n = 5$, $P < 0.01$).

We also tested the effect of 5-HT treatment on the production of $O_2^{-\bullet}$ using a cytochrome *c* reduction assay [25] (Figure 2B). In their unstimulated state, CHO cells produced ≈ 75 pmoles of $O_2^{-\bullet}$/min/10^6 cells (≈ 400 pmoles of $O_2^{-\bullet}$/min per mg of protein), and this was doubled to ≈ 160 pmoles of $O_2^{-\bullet}$/min/10^6 cells (≈ 850 pmoles of $O_2^{-\bullet}$/min per mg of protein) after treatment with 1 μM 5-HT. This effect was blocked by DPI, and suppressed below baseline by including superoxide dismutase (which converts of $O_2^{-\bullet}$ into H_2O_2) in the assay mixture. To determine whether this conversion occurs in CHO cells, cells were incubated with DCF-DA to detect the production of intracellular H_2O_2. 5-HT induced a rapid increase in intracellular fluorescence, which could be blocked by NAC and by DPI, suggesting that 5-HT induces production of H_2O_2 through NAD(P)H oxidase (Figure 3).

NAD(P)H oxidase inhibitors block activation of ERK

NAD(P)H oxidase is a multi-component enzyme that catalyses the transfer of electrons from NADH and NADPH to O_2, resulting in the production of $O_2^{-\bullet}$ [30]. We used four types of pharmacological inhibitors of NAD(P)H oxidase to test its involvement in the activation of ERK by 5-HT. 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 [31,32]. DPI blocks FAD binding to a flavin site of the oxidase [31]. PAO blocks distal electron transport in the NAD(P)H oxidase [33]. AEBSF is a serine protease inhibitor that blocks assembly of the NAD(P)H oxidase enzyme subunits; the serine protease inhibitory properties of AEBSF do not appear to be required for blockade of NAD(P)H oxidase [34].

Figure 4(A) shows that 100 μM HMAP, 50 μM DPI and 1 μM PAO each markedly attenuated the activation of ERK by 5-HT. The effect of 1 μM PAO could be reversed by co-incubation with 20 μM BAL, as has been shown to occur with purified oxidase components [32]. A series of compounds with varying degrees of structural similarities to AEBSF was also tested for their ability to inhibit the activation of ERK by 5-HT. As shown in Figure 4(B), MAEBSF, which shares the major structural features of AEBSF, also inhibited ERK phosphorylation [34]. Neither

AEBSAC [4-(2-aminoethyl)-benzenesulphonic acid] in which the sulphonyl fluoride moiety is substituted with a sulphonic acid, nor PMSF, which preserves only the sulphonyl fluoride moiety, were able to inhibit ERK phosphorylation. Similarly, $AEBSNH_2$, in which the sulphonyl fluoride moiety is substituted with a sulphonamide had no effect (results not shown, $n = 4$). A structurally unrelated serine protease inhibitor, TLCK, had no effect (Figure 4B). These studies provide pharmacological evidence for the specific involvement of an NAD(P)H oxidase enzyme in the activation of ERK.

Mapping studies

We assessed the ability of antioxidants to block the phosphorylation of MEK, which resides one step upstream of ERK in the signalling cascade. Because these experiments showed that antioxidants block MEK phosphorylation to the same degree that they block ERK phosphorylation (results not shown, $n = 3$), it was likely that the ROS act at a point upstream of MEK. Next, constitutively activated Ras, Raf or MEK were transfected into cells, followed by treatment with antioxidants. These experiments showed no effect of NAC or DPI on Ras-, Raf- or MEK-induced ERK phosphorylation (Figure 5A). The above experiments confirm a location of the ROS upstream of MEK, and further suggest that the ROS intersect at or before the level of Ras. Next, we used dominant negative constructs of Ras and Raf, and a chemical inhibitor of MEK to attenuate the phosphorylation of ERK induced by H_2O_2. These experiments showed that both constructs and PD98059 attenuated the H_2O_2 response, supporting a location for the ROS that is upstream of Ras (Figure 5B). NAC and DPI were ineffective in blocking the activation of ERK induced by transfection with constitutively activated Src, suggesting that Src resides downstream of the ROS (Figure 5C). We used a Src inhibitor compound, PP1, to confirm that the relative location of Src is downstream from the ROS. PP1 (10 μM for 60 min) greatly attenuated the phosphorylation of ERK induced by 1 μM 5-HT ($80 \pm 2\,\%$ inhibition, results not shown), by 100 nM 8-hydroxy-2-(di-*n*-propylamino)-tetralin (specific 5-HT_{1A} receptor agonist; $94 \pm 1\,\%$ inhibition, results not shown), and by 10 and 100 μM H_2O_2 (Figures 5D and 5E). In other studies (results not shown), PP1 did not block the increase in DCF-DA fluorescence induced by 5-HT. In aggregate, the studies demonstrate a location for the ROS upstream of Src in the G$\beta\gamma$ pathway.

DISCUSSION

In the current study, we attempted to delineate the major point of interaction of ROS in a well-defined model system (the CHO cell), which utilizes a transfected 5-HT_{1A} receptor that couples exclusively to pertussis toxin-sensitive G proteins. There are two new findings presented in this work. The major point of intersection of ROS is upstream of Src in the $G_i\beta\gamma \rightarrow$ ERK pathway; and an NAD(P)H oxidase-like enzyme appears to mediate the production of the ROS in the $G_i\beta\gamma \rightarrow$ ERK pathway. Our results also fulfil five key criteria that support a major role for ROS in the activation of ERK. First, antioxidants were shown to inhibit activation of ERK. Secondly, direct application of cysteine-reactive oxidant molecules activated ERK. Thirdly, the 5-HT_{1A} receptor was shown to alter cellular reducing rate by redox microphysiometry, and to generate both $O_2^{-\bullet}$ and H_2O_2. The amount of $O_2^{-\bullet}$ produced was significant, equalling that which has been described in activated VSMC [35], and being about one-fifth to one-twentieth of that described in neutrophils [33,36] and macrophages [34]. Fourthly, there was evidence that a specific ROS-producing enzyme [NAD(P)H oxidase] is involved in the activation of ERK. Fifthly, there was specificity both in the effects of various chemical oxidizers, and in the putative location of the ROS in the ERK activation pathway.

On a molecular level, there are multiple potential targets for the actions of ROS, and many of these are important in the activation of ERK. At least five types of targets (tyrosine kinases, tyrosine phosphatases, Ras, Raf and PKC) in the ERK pathway have been shown to be sensitive to various ROS. Tyrosine kinases can be activated by oxidation of critical thiol groups [37], and several Src family kinase members can be activated by H_2O_2 and diamide [27]. We previously showed that ERK activation by the 5-HT_{1A} receptor in CHO cells could be blocked by transfection with an inhibitor of Src (Csk) [22], and preliminary studies showed that the 5-HT_{1A} receptor in CHO cells could activate Src-like kinase activity to phosphorylate the peptide KVEKIGEGTYGVVYK (cdc2 residues 6–20) by 2.3-fold within 1 min ($n = 3$, results not shown). The gastrin/cholecystokinin receptor has also been shown to activate Src in CHO cells [38]. In renal mesangial cells, oxidative stress results in tyrosine phosphorylation of pp60c-src and PDGF receptors [39]. Thus there is rationale for positing a role for $G_i\beta\gamma$-generated ROS acting at the level of, or upstream of Src. Our results were not predictable, as one group was not able to show a role for Shc, which is phosphorylated by Src, in the activation of ERK by ROS [1], and another group presented evidence that Src is not involved in $\beta\gamma$-mediated activation of ERK [40]. Moreover, Cunnick et al. [40a] showed that H_2O_2 negatively regulates Src in HeLa cells [9]. Although our results support a role for Src as a target of ROS, they do not allow a distinction between a direct activation of Src and an inactivation of a tyrosine phosphatase at or above the level of Src in CHO cells. In that regard, H_2O_2 can up-regulate MKP-1, a protein tyrosine phosphatase that specifically dephosphorylates and inactivates ERK [3]. On the other hand, H_2O_2 and diamide have been shown to rapidly inactivate MKP-1 in neutrophils [1]. The known protein tyrosine phosphatases nearly all have reactive cysteine residues in their active sites [41–43], and this site is particularly susceptible to oxidation due to its low pK_a (< 5) [44]. H_2O_2 has been shown to directly inhibit protein tyrosine phosphatase activity *in vitro*, and this response can be reversed by treatment with the reducing agent, dithiothreitol [45]. Because the *in vitro* specific activities of tyrosine phosphatases are 10–1000 times greater than those of tyrosine kinases [46], they would be ideal targets for regulation by ROS. Nevertheless, our data support a location of the ROS at the level of, or upstream of Src.

Other potential targets for ROS are PKC [3,17], Ras [47], and Raf [48]. Previous studies have ruled out a significant role for PKC in signalling by the 5-HT_{1A} receptor in these cells [21,22], and the current work does not support a significant role for Ras or Raf as targets of ROS (Figure 5). Our results support a key role for an NAD(P)H oxidase-like enzyme as the source of the ROS, which induce the phosphorylation of ERK. This does not appear to be a peculiarity of the CHO cell system in that 64 % of ERK activation induced by the 5-HT_{1A} receptor in transfected HEK 293 cells can be blocked by antioxidants ($n = 3$, results not shown), although we have not yet performed similar studies in cells that naturally express the 5-HT_{1A} receptor. There is precedent that activation of NAD(P)H oxidase and the subsequent generation of ROS mediates proliferative signals in VSMC [48,49] and renal mesangial cell [50], although recent results has been presented that do not support a specific role for NAD(P)H oxidase in activation of ERK in VSMC [9]. In contrast, the present work supports a role for NAD(P)H oxidase in the ERK activation pathway, and that this occurs at a point upstream of

Src. It is also likely that the enzyme is located downstream of $G_i\beta\gamma$, because the G proteins are thought to be the most proximal transducers of receptor signals. Therefore, we propose that NAD(P)H oxidase is located in the ERK activation pathway stimulated by the transfected 5-HT_{1A} receptor in CHO cells as follows: 5-HT_{1A} receptor → $G_i\beta\gamma$ subunits → NAD(P)H oxidase → ROS → Src → Shc → Grb2 → Sos → Ras → Raf → MEK → ERK. The precise mechanisms through which $G_i\beta\gamma$ subunits activate NAD(P)H oxidase, and through which ROS activate Src, and which specific ROS mediates the activation of the ERK pathway remain to be clarified. Moreover, the relationship of NAD(P)H oxidase and ROS to other unusual signals linked to ERK activation (such as endocytosis [51]), also remain to be clarified.

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