Biochemical Pharmacology, Vol. 56, pp. 527–533, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(98)00174-9

Superoxide Scavenging Effect of *Ginkgo biloba* Extract on Serotonin-induced Mitogenesis

Sheu-Ling Lee, Wei-Wei Wang, Joseph Lanzillo, C. Norman Gillis and Barry L. Fanburg*

Pulmonary and Critical Care Division/Tupper Research Institute, Department of Medicine, New England Medical Center/Tufts University School of Medicine, Boston, MA, U.S.A.

ABSTRACT. We have reported previously that serotonin (5-HT) stimulates the mitogenesis of bovine pulmonary artery smooth muscle cells (SMCs) through active transport of 5-HT and cellular signaling that includes elevation of superoxide ($O_2^{\cdot -}$) and enhancement of protein tyrosine phosphorylation. *Ginkgo biloba* extract 501 (EGb 501), which has been demonstrated to act as an antioxidant, was found to block both the elevated $O_2^{\cdot -}$ and the proliferative and hypertrophic influences of 5-HT on SMCs, but not to directly inhibit the associated activation of NAD(P)H oxidase or the stimulation of phosphorylation of GTPase-activating protein (GAP). A similar effect of *Ginkgo biloba* extract 501 occurred on Chinese hamster lung fibroblasts (CCL-39), where 5-HT receptor, as opposed to transporter, action has been associated with mitogenesis. We conclude from these studies that *Ginkgo biloba* extract 501 quenches $O_2^{\cdot -}$ formation by 5-HT, thereby blocking its mitogenic effect. Stimulation of protein tyrosine phosphorylation of GAP by 5-HT appears to precede the elevation of $O_2^{\cdot -}$. BIOCHEM PHARMACOL **56**;4:527–533, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. 5-HT; *Ginkgo biloba*; superoxide; cellular hyperplasia and hypertrophy; antioxidant; tyrosine phosphorylation

5-HT† has been increasingly recognized to be a mitogen for both vascular and non-vascular cells [1]. For bovine pulmonary artery SMCs, we have shown previously that through its active transport [2] 5-HT rapidly induces tyrosine phosphorylation of GAP, possibly activates $p21^{ras}$ [3], and produces cellular hyperplasia and hypertrophy [4, 5]. We recently reported that 5-HT produces a rapid elevation of $O_2^{\cdot -}$ that mediates the mitogenic signal of 5-HT on SMCs [6]. The $O_2^{\cdot -}$ free radical quencher 4,5-dihydroxy-1,3-benzene disulfonic acid (Tiron) and *N*-acetyl-L-cysteine (NAC), which provides cysteine as a precursor for cellular glutathione, block both the 5-HT-induced formation of $O_2^{\cdot -}$ and cellular proliferation.

Ginkgo biloba extract, commonly used in Europe to treat peripheral arterial diseases and cerebral insufficiency [7], also has been used to protect against ischemia–reperfusion injury, and the protection has been attributed to its antioxidant properties [8, 9]. This extract has been reported to be a potent scavenger of superoxide [10, 11], hydroxyl [12], and peroxyl [13] radicals and NO [11]; it also inhibits the enzymatic activity of inducible nitric oxide synthase [14]. The data presented in this report show that EGb 501 scavenges $O_2^{\cdot -}$ as effectively as other antioxidants in an *in vitro* system and blocks 5-HT-induced $O_2^{\cdot -}$ elevation and mitogenesis of both SMCs and fibroblasts. To our knowledge, this is the first report to demonstrate that *G. biloba* extract inhibits a cellular transduction signaling process that leads to mitogenesis through its ability to quench $O_2^{\cdot -}$.

MATERIALS AND METHODS

Reagents

EGb 501 (Batch No. 91196) was a gift from Dr. Fabio Soldati, Pharmaton SA, to Dr. Gillis. DPI was obtained from ICN Pharmaceuticals. α-Hydroxyfarnesylphosphonic acid was from Biomol Research Laboratories. All other reagents were from the Sigma Chemical Co.

Cell Culture

SMCs from bovine pulmonary arteries were isolated and cultured by a modification of the method of Ross [15] as previously described [2]. In these experiments, 3rd to 5th passage SMCs were used. Chinese hamster lung fibroblasts (CCL-39) were obtained from the American Type Culture Collection and cultured in McCoy's 5A medium with 10% FBS.

* Corresponding author: Sheu-Ling Lee, Ph.D., New England Medical Center, Pulmonary and Critical Care Division, 750 Washington St., NEMC #265, Boston, MA 02111. Tel. (617) 636-5874; FAX (617) 636-5953.

† *Abbreviations:* 5-HT, serotonin; DPI, diphenyliodonium; EGb 501, *Ginkgo biloba* extract 501; FBS, fetal bovine serum; GAP, GTPase-activating protein; $O_2^{\cdot -}$, superoxide; and SMCs, smooth muscle cells.

Received 15 January 1998; accepted 24 April 1998.

Incorporation of [^{3}H]Thymidine

The protocol used has been described in detail [2]. In brief, plated SMCs were cultured for 72 hr in RPMI medium containing 10% FBS followed by 72-hr growth arrest in medium containing 0.1% FBS. Then SMCs were incubated at a density of 0.1×10^6 cells/35 mm Petri dish with and without 1 μM 5-HT in the same medium for 20 hr before being labeled with [*methyl*-^{3}H]thymidine (0.1 mCi/mL, specific activity 20 Ci/mmol, New England Nuclear) for 4 hr. Iproniazid (an inhibitor of degradation of 5-HT by monoamine oxidase) or other inhibitors were added 30 min before the 5-HT. These agents alone at the concentrations reported did not alter the incorporation of [^{3}H]thymidine by SMCs. After labeling, experiments were terminated by aspiration of medium and washing the cellular monolayer first with ice-cold PBS and then with cold 6% trichloroacetic acid. Then cells were dissolved in 0.2 N NaOH, and radioactivity was counted in a liquid scintillation counter (model 1219, Pharmacia LKB Biotechnology). The only modification to this protocol used for CCL-39 was that the cells were growth arrested in McCoy's medium with 0.01% FBS for 48 hr followed by incubation with or without 5-HT in the presence or absence of inhibitors for 18 hr and labeled with [^{3}H]thymidine (0.5 mCi/mL) for the final 3 hr.

Cell Number and Size Analysis

At the end of the incubation period, control and treated cells were trypsinized and diluted in Isoton (Stephens Scientific). Cell number and size distribution measurements were carried out with the use of a Coulter counter equipped with a Coulter Channelyzer and an x-y Recorder 4 (Coulter), as previously described [4]. Changes in cellular size were determined and expressed as percent change from the control.

Measurement of Superoxide Anion Production in Intact Cells by a Lucigenin-enhanced Chemiluminescence (LUCL) Assay [6, 16]

SMCs were cultured in 100-mm Petri dishes and growth arrested for 72 hr as previously described [2]. Then cells were trypsinized, pelleted by centrifugation, and resuspended at a concentration of 10^6 cells/mL in PBS containing 10 mM glucose and 1 mg/mL of bovine serum albumin. For experimental samples, 5-HT and other reagents were added to the cellular suspension in the cuvette and incubated at 37° for the period of time noted in Results. In the cases where longer incubation periods with reagents were needed (>3 hr), 5-HT and other reagents were first added directly to the cellular monolayer. Then cells were trypsinized, pelleted by centrifugation, and resuspended in PBS as noted above. 5-HT and/or other reagents were then added again to the cellular suspensions in the cuvette, after which the suspensions were loaded into a luminometer, and lucigenin (final concentration 500 μM) was injected automatically to start the reaction. A 15-sec dark-adapted period was carried out prior to each sample reading in the luminometer. Photoemission was recorded with 60 sec integration for 10 min with a Lumac Biocounter M2010 (Lumac System, Inc.). Buffer blank or lucigenin or other reagents alone used in these studies produce negligible chemiluminescence. Data are expressed as counts of chemiluminescence per 10^6 cells or fold stimulation of chemiluminescence relative to control.

NAD(P)H Oxidase Measurement

SMCs were cultured and treated as described above for the LUCL assay. NADH/NADPH activity was measured by a luminescence assay as described by Griendling *et al.* [17] except that 10 μM NAD(P)H substrate was used. NADH/NADPH alone did not produce any chemiluminescence.

Preparation of Whole Cell Extracts for Electrophoresis

Cells were grown in 100-mm Petri dishes to confluency and were growth arrested in medium with 0.1% FBS for 72 hr. The cells were preincubated with inhibitors for 30 min prior to the addition of 1 μM 5-HT for 30 and 60 min; then cellular monolayers were washed twice with ice-cold PBS. Cell lysates were obtained by incubating the cellular monolayer in 1 mL of cell lysis buffer [18] containing 50 mM Tris–HCl, pH 7.5, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM sodium molybdate, 10 μg/mL of aprotinin, 10 μg/mL of leupeptin, 10 μg/mL of soybean trypsin inhibitor, 40 μg/mL of phenylmethylsulfonyl fluoride (PMSF), 0.07 μg/mL of pepstatin, 1% Nonidet P-40, 150 mM NaCl, and 5 mM EDTA for 10 min at 4°. The insoluble material was removed by centrifugation (14,000 *g*, 2 min), and the supernatant fraction was used for analysis. Twenty to fifty micrograms of protein of the whole cell lysate was subjected to SDS–PAGE on an 8.5% slab gel in a model SE-600 apparatus (Hoefer Scientific) according to Laemmli [19].

Immunoblotting with Anti-phosphotyrosine Antibody

After electrophoresis, gel proteins were transferred electrophoretically to a polyvinylidene disfluoride (PVDF) membrane (Tropifluor, Tropix, or Immobilon-P, Millipore). After transfer, nonspecific PVDF binding sites were blocked with 5% HiPure liquid gelatin (Norland) in buffer, pH 7.4, containing 75 mM sodium phosphate, 70 mM NaCl, 0.02% sodium azide, and 0.1% Tween 20 [20]. Blocking was done for 1 hr at ambient temperature. The membrane was then treated with a 1:2500 dilution of alkaline phosphatase-conjugated anti-phosphotyrosine antibody in blocking buffer for 90 min at ambient temperature with gentle agitation. Nitro-block (Tropix) was used according to the manufacturer's instructions. Subsequent washing, detection with the chemiluminescent substrate

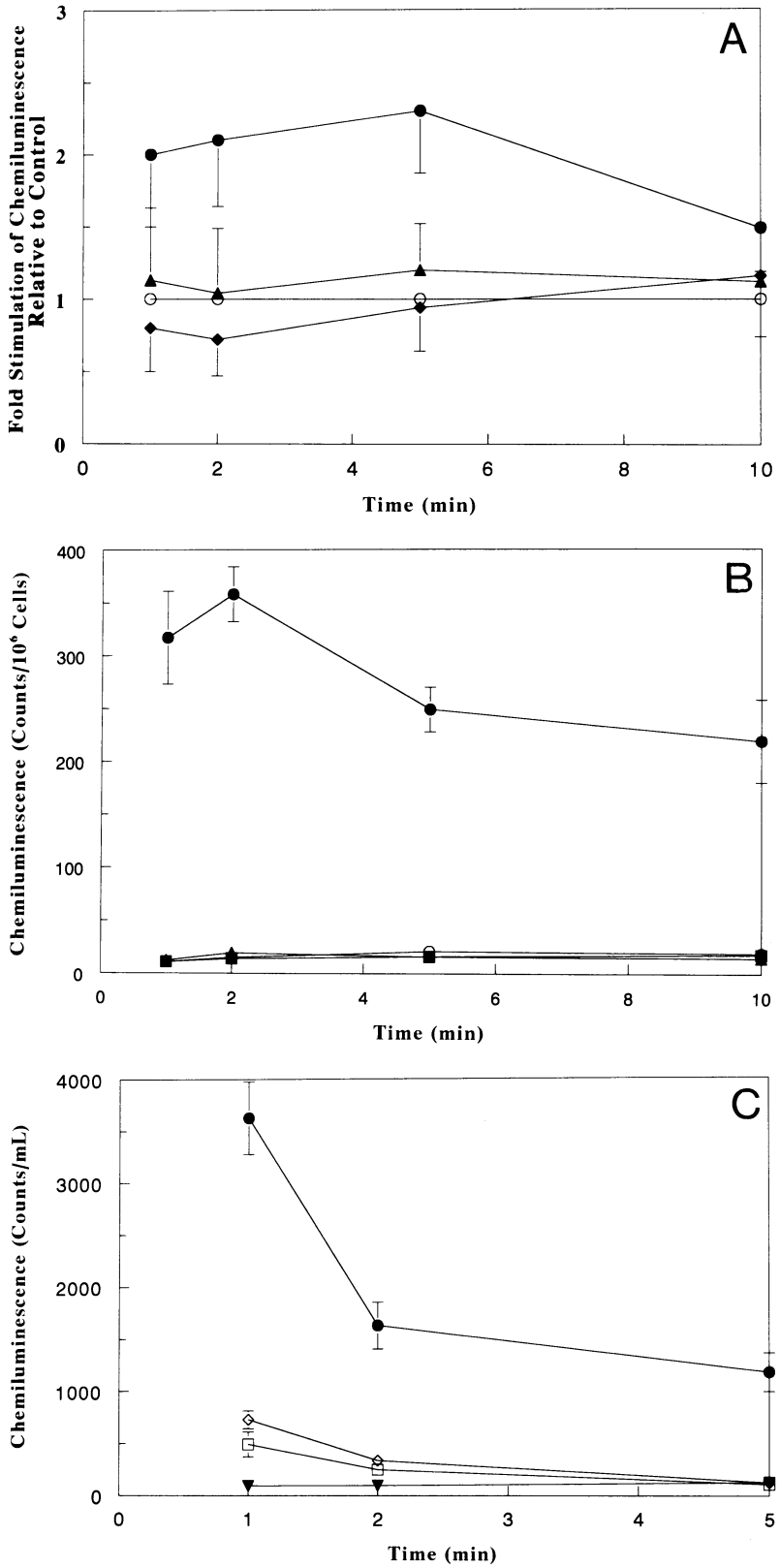

FIG. 1. Superoxide scavenging effect of EGb 501. (A) Blockade by EGb 501 (100 μg/mL, ▲) and α-hydroxyfarnesylphosphonic acid (30 μM, ◆) of the induction of $O_2^{\cdot -}$ generated by a 1-hr incubation of SMCs with 1 μM 5-HT (●). Control, ○. (B) Effects of EGb 501 (100 μg/mL, ▲) and SOD (150 U/mL, ■) on the $O_2^{\cdot -}$ generated from SMCs incubated with menadione (10 μM, ●) for 5 min. Control, ○. (C) Abolition by EGb 501 (100 and 200 μg/mL, ◇ and □, respectively) of the $O_2^{\cdot -}$ generated from the interaction of xanthine (60 μM) with xanthine oxidase (2 mU/mL) (●). The effect of 150 U/mL of SOD (▼) is also shown. Photoemission was recorded as described in Materials and Methods at the times indicated after the addition of lucigenin. Values are means ± SD (N = 4). Some of the error bars are too small to be visible.

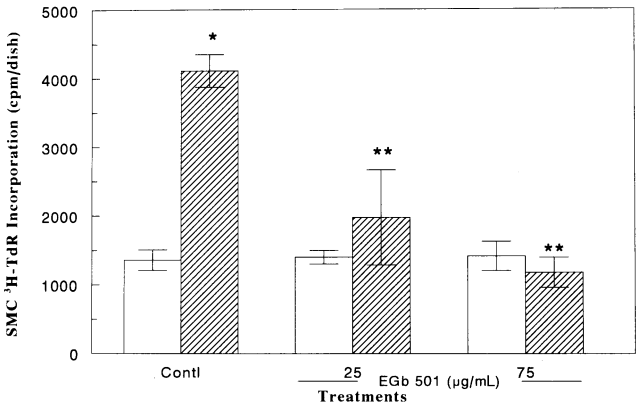

FIG. 2. Effect of EGb 501 on 1 μM 5-HT-stimulated DNA synthesis of SMCs. (Control, □; +5-HT, ▨.) Values are means ± SD (N = 4). *P < 0.05, significantly different from control; and **P < 0.05, significantly different from 5-HT alone.

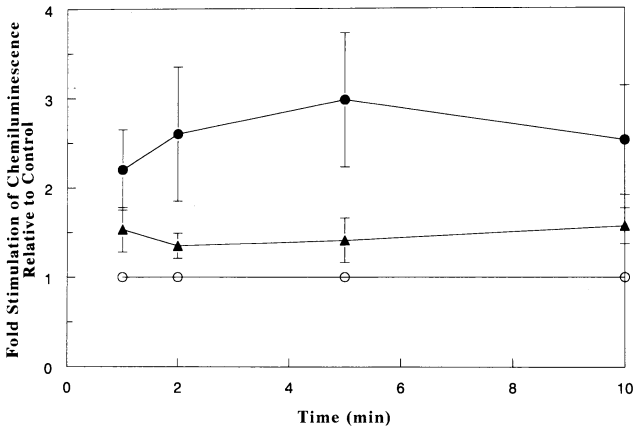

FIG. 4. Superoxide production from intact CCL-39 after incubation with 1 μM 5-HT (●) for 18 hr. Superoxide production was assayed by lucigenin photoemission as described for Fig. 1. Other symbols: (▲) EGb 501 (100 μg/mL), and (○) control. Values are means ± SD (N = 4).

CSPD (Tropix), and film exposure were done as described previously [20].

RESULTS

Serotonin induced a 2- to 3-fold elevation of $O_2^{\cdot -}$ formation from SMCs after 1 hr of incubation, and this elevation was totally abolished by 100 μg/mL of EGb 501 (Fig. 1A). Superoxide generated from other sources such as the action of menadione (10 μM) on SMCs for 5 min (Fig. 1B) and the interaction of xanthine with xanthine oxidase (60 μM and 2 mU/mL, respectively) was also totally scavenged by EGb 501, as well as inactivated by 150 U/mL of superoxide dismutase (SOD) (Fig. 1, B and C). Other antioxidants such as 4,5-dihydroxy-1,3-benzene disulfonic acid (Tiron) and *N*-acetyl-L-cysteine (NAC) also block $O_2^{\cdot -}$ formation [6]. Thus, EGb 501 serves as a quenching agent for $O_2^{\cdot -}$ produced from either stimulation of SMCs by 5-HT, intracellular oxidation of menadione, or interaction of xanthine and xanthine oxidase. Furthermore, inhibition of $p21^{ras}$ activation with 30 μM α-hydroxyfarnesylphosphonic acid [21] for 1 hr blocked 5-HT-induced $O_2^{\cdot -}$ formation in SMCs (Fig. 1A).

We have shown previously that $O_2^{\cdot -}$ acts as an inter-

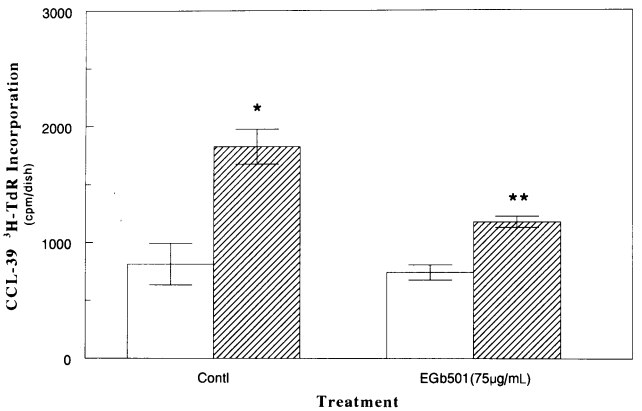

FIG. 3. Effect of EGb 501 on 1 μM 5-HT-stimulated DNA synthesis of CCL-39. (Control, □; +5-HT, ▨.) Values are means ± SD, N = 4. *P < 0.05, significantly different from control; and **P < 0.05, significantly different from 5-HT alone.

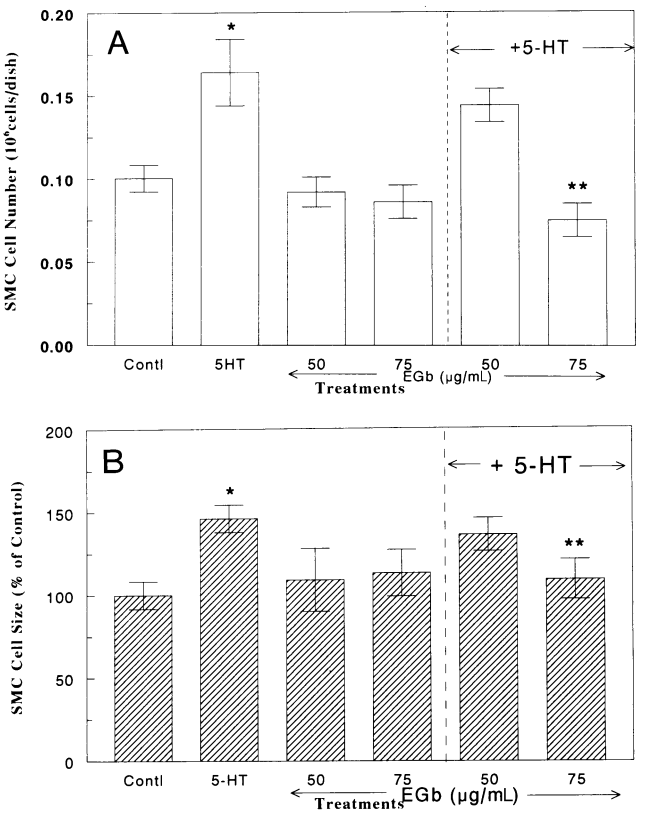

FIG. 5. Effects of EGb 501 on 5-HT-induced hyperplasia (A) and hypertrophy (B) of SMCs. Cells were incubated for 7 days with 1 μM 5-HT with and without 50 or 75 μg/mL of EGb 501. Values are means ± SD (N = 4). *P < 0.05, significantly different from control; and **P < 0.05, significantly different from 5-HT alone.

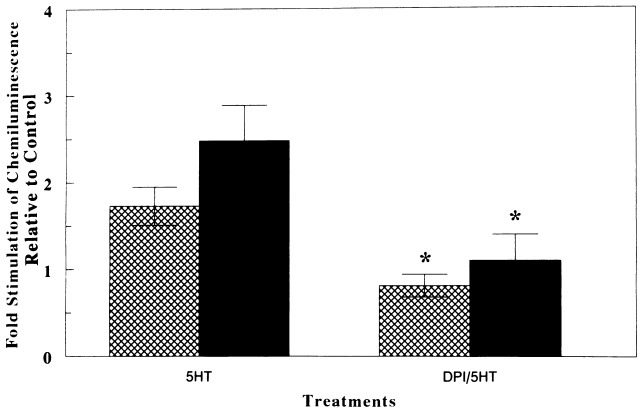

FIG. 6. Effect of DPI on NAD(P)H oxidase. Cells were incubated with 1 μM 5-HT for 30 min to determine its effect on the activities of both NADH (▨) and NADPH (■) oxidase of SMCs. Another two groups of cells were preincubated for 1 hr with 100 μM DPI prior to the addition of 5-HT. NADH and NADPH (each at 10 μM) were used as substrates. Data are photoemission recordings at a 5-min time point. Values are means ± SD (N = 4). *P < 0.05, significantly different from 5-HT alone.

mediate in the mitogenic action of 5-HT on SMCs [6]. In addition to blocking $O_2^{\cdot -}$ formation by 5-HT, EGb 501 blocked the stimulatory action of 5-HT on [^{3}H]thymidine incorporation by SMCs (Fig. 2). Similar to its effect on SMCs, 5-HT stimulated cellular proliferation and $O_2^{\cdot -}$ formation of CCL-39 that were blocked by EGb 501 (Figs. 3 and 4).

Serotonin not only stimulates [^{3}H]thymidine incorporation by SMCs but also elevates SMC number and produces cellular hypertrophy [4]. Like its effect on [^{3}H]thymidine incorporation, EGb 501 blocked the 5-HT stimulatory effect on SMC number and size (Fig. 5, A and B).

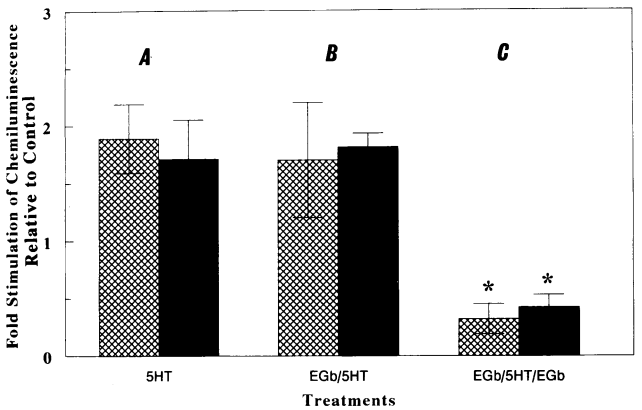

FIG. 7. NADH (▨) and NADPH (■) oxidase activities of SMCs. Chemiluminescence experiments were carried out as noted in Materials and Methods. Section A: 1 μM 5-HT alone for 30 min. Section B: EGb 501 (100 μg/mL) preincubated for 1 hr prior to the addition of 5-HT. Section C: as in Section B except that 100 μg/mL of EGb 501 was re-added into the cuvette prior to the chemiluminescence reading. Data are photoemission recordings at a 5-min time point. Values are means ± SD (N = 4). *P < 0.05, significantly different from 5-HT alone.

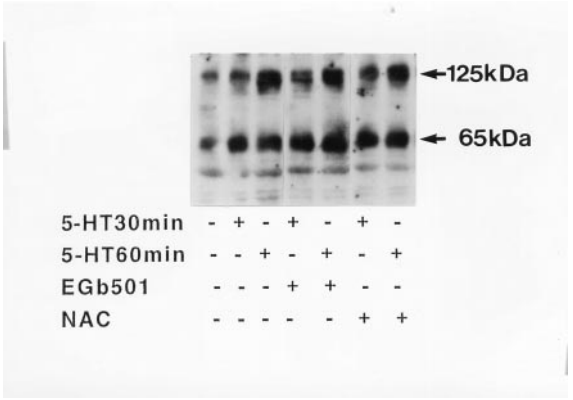

FIG. 8. Tyrosine phosphorylation of GAP (p120) and co-precipitated p65. Cells were treated with 1 μM 5-HT for 30–60 min in the presence and absence of 100 μg/mL of EGb 501 or 10 mM NAC.

We next explored possible actions other than $O_2^{\cdot -}$ quenching through which EGb 501 might inhibit the growth stimulatory effect of 5-HT. Because we had found previously that DPI, a compound that forms covalent adducts with flavin moieties and inhibits flavin-linked oxidases [22], blocks SMC $O_2^{\cdot -}$ elevation by 5-HT [6], we tested the effect of 5-HT on $O_2^{\cdot -}$ formation in the presence of added NADH and NADPH substrates. Figure 6 shows that the stimulation of $O_2^{\cdot -}$ by 5-HT was enhanced in the presence of both of these substrates. The stimulation of $O_2^{\cdot -}$ by 5-HT in the presence of added NADH and NADPH substrate was also inhibited by DPI (Fig. 6). We considered the possibility that EGb 501 might inhibit 5-HT-stimulated $O_2^{\cdot -}$ formation through a direct structural action on NAD(P)H oxidase activity. However, in experiments in which cultured SMCs were first treated with EGb 501 for 1 hr and medium containing EGb 501 was removed prior to the addition of NAD(P)H substrate to the cuvette, there was no inhibition of $O_2^{\cdot -}$ formation (Fig. 7, B vs A). On the other hand, when EGb 501 was re-added to the suspended cells in the cuvette as $O_2^{\cdot -}$ was being formed, there was a marked inhibition of chemiluminescence (Fig. 7C). Thus, these data support a concept that EGb 501 quenches $O_2^{\cdot -}$, but does not produce a direct inhibition of the NAD(P)H oxidase enzyme itself.

We have found that protein tyrosine phosphorylation and, in particular, that of GAP is stimulated in SMCs by 5-HT [5]. Tyrosine phosphorylation of protein appears to be important in the induction of the cellular proliferative process. Thus, we reasoned that EGb 501 might block $O_2^{\cdot -}$ formation and the cellular proliferative process through its inhibition of protein tyrosine phosphorylation. However, EGb 501 had no effect on tyrosine phosphorylation of GAP (Fig. 8). NAC, likewise, had no effect on the stimulation of protein tyrosine phosphorylation of GAP by 5-HT. The results of these studies suggest that protein tyrosine phosphorylation of GAP precedes the production of $O_2^{\cdot -}$ by 5-HT and are consistent with an inhibition of $O_2^{\cdot -}$ formation by EGb 501 through its quenching effect.

DISCUSSION

We have accumulated evidence showing that 5-HT (1 μM) stimulates both hyperplasia and hypertrophy of SMCs in culture [4]. For bovine pulmonary artery SMCs, the stimulation is dependent upon the active transport of 5-HT and involves the enhancement of protein phosphorylation. In particular, GAP (p120) and p65, which co-precipitates with p120 [23], show early enhanced phosphorylation [5]. Our recent studies showing that stimulation of $O_2^{\cdot -}$ formation is an intermediary step in the induction of cellular proliferation by 5-HT [6] parallel other studies indicating that reactive oxygen intermediates act as second messengers in stimulating cellular proliferation [24, 25]. Furthermore, our data demonstrated that 5-HT elevates $O_2^{\cdot -}$ formation through activating NAD(P)H oxidase, similar to other observations with angiotensin II-induced rat aorta SMC hyperplasia/hypertrophy [17].

While searching for agents that might influence the 5-HT-induced SMC proliferative process, we considered EGb 501, since it is known to have antioxidant properties due to its flavonoid constituents [10, 26–28]. Indeed, EGb 501 blocked 5-HT stimulation of proliferation of both bovine pulmonary artery SMCs and Chinese hamster lung fibroblasts (CCL-39), and all of our studies suggest that the inhibitory effect is through the $O_2^{\cdot -}$ scavenging capability of EGb 501. No direct inhibitory effect by EGb 501 was observed on either NAD(P)H oxidase activity or protein tyrosine phosphorylation of GAP, intermediary processes in the stimulatory effect.

It is of interest that 5-HT produced EGb 501-inhibitable stimulation of proliferation of CCL-39 as well as that of the SMCs. Unlike the SMCs whose proliferative process has been related to 5-HT transport [2], mitogenesis of CCL-39 has been reported to occur through activation of 5-HT_{1B} receptors [29]. Hence, downstream stimulatory pathways for 5-HT may be similar for cells whether they are activated through a 5-HT transporter or a 5-HT receptor.

EGb 501 may also serve as a useful tool to dissect the sequence of the pathway through which 5-HT produces mitogenesis. It is now known that ras protein, a key regulator of cellular growth, requires farnesylation for its activation [21]. A $p21^{ras}$ inhibitor (α-hydroxyfarnesylphosphonic acid) dose-dependently reversed both the 5-HT-induced [^{3}H]thymidine incorporation and stimulation of $O_2^{\cdot -}$ formation [6]. Because EGb 501 blocked $O_2^{\cdot -}$ formation and cellular proliferation produced by 5-HT in our studies, yet had no effect on NAD(P)H oxidase activity or protein tyrosine phosphorylation of GAP of cells, it is likely that the $O_2^{\cdot -}$ formation follows, rather than precedes, the stimulation of tyrosine phosphorylation. Our current findings are consistent with previous studies showing that $p21^{ras}$ activation is required for 5-HT-induced cellular proliferation [6] and are consonant with the finding of inhibition of $O_2^{\cdot -}$ formation and cellular proliferation by α-hydroxyfarnesylphosphonic acid, a selective inhibitor of farnesyl-protein transferase, which blocks ras activation [21].

This study was supported by Research Grant HL 32723 from the Heart, Lung and Blood Institute of the National Institutes of Health.

References

1. Fanburg BL and Lee SL, A new role for an old molecule: Serotonin as a mitogen. *Am J Physiol* **272**(5 Pt 1)**:** L795–L806, 1997.
2. Lee SL, Wang WW, Moore BJ and Fanburg BL, Dual effect of serotonin on growth of bovine pulmonary artery smooth muscle cells in culture. *Circ Res* **68:** 1362–1368, 1991.
3. Satoh T, Endo M, Nakafuku M, Akiyama T, Yamamoto T and Kaziro Y, Accumulation of $p21^{ras}$ · GTP in response to stimulation with epidermal growth factor and oncogene products with tyrosine kinase activity. *Proc Natl Acad Sci USA* **87:** 7926–7929, 1990.
4. Lee SL, Wang WW, Lanzillo JJ and Fanburg BL, Serotonin produces both hyperplasia and hypertrophy of bovine pulmonary artery smooth muscle cells in culture. *Am J Physiol* **266**(1 Pt 1)**:** L46–L52, 1994.
5. Lee SL, Wang WW and Fanburg BL, Association of Tyr phosphorylation of GTPase-activating protein with mitogenic action of serotonin. *Am J Physiol* **272**(1 Pt 1)**:** C223–C230, 1997.
6. Lee SL, Wang WW and Fanburg BL, Superoxide as an intermediate signal for serotonin-induced mitogenesis. *Free Radic Biol Med* **24:** 855–858, 1998.
7. DeFeudis FV, Ginkgo biloba *Extract (EGb 761): Pharmacological Activities and Clinical Application*. Elsevier, Paris, 1991.
8. Haramaki N, Aggarwal S, Kawabata T, Droy-Lefaix MT and Packer L, Effects of natural antioxidant *Ginkgo biloba* extract (EGb 761) on myocardial ischemia–reperfusion injury. *Free Radic Biol Med* **16:** 789–794, 1994.
9. Szabo ME, Droy-Lefaix MT and Doly M, Modification of reperfusion-induced ionic imbalance by free radical scavengers in spontaneously hypertensive rat retina. *Free Radic Biol Med* **13:** 609–620, 1992.
10. Pincemail J, Dupuis M, Nasr C, Hans P, Haag-Berrurier M, Anton R and Deby C, Superoxide anion scavenging effect and superoxide dismutase activity of *Ginkgo biloba* extract. *Experientia* **45:** 708–712, 1989.
11. Marcocci L, Packer L, Droy-Lefaix M-T, Sekaki A and Gardes-Albert M, Antioxidant action of *Ginkgo biloba* extract EGb 761. *Methods Enzymol* **234:** 462–475, 1994.
12. Gardes-Albert M, Ferradini C, Sekaki A and Droy-Lefaix MT, Oxygen-centered free radicals and their interactions with EGb 761 or CP 202. In: *Advances in* Ginkgo biloba *Extract Research* (Eds. Ferredini C, Droy-Lefaix MT and Christen Y), pp. 1–11. Elsevier, Paris, 1993.
13. Maitra I, Marcocci L, Droy-Lefaix MT and Packer L, Peroxyl radical scavenging activity of *Ginkgo biloba* extract EGb 761. *Biochem Pharmacol* **49:** 1649–1655, 1995.
14. Kobuchi H, Droy-Lefaix MT, Christen Y and Packer L, *Ginkgo biloba* extract (EGb 761): Inhibitory effect on nitric oxide production in the macrophage cell line RAW 264.7. *Biochem Pharmacol* **53:** 897–903, 1997.
15. Ross R, The smooth muscle cell. II. Growth of smooth muscle in culture and formation of elastic fibers. *J Cell Biol* **50:** 172–186, 1971.
16. Gyllenhammar H, Lucigenin chemiluminescence in the assessment of neutrophil superoxide production. *J Immunol Methods* **97:** 209–213, 1987.

17. Griendling KK, Minieri CA, Ollerenshaw JD and Alexander RW, Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res* **74:** 1141–1148, 1994.
18. Welham MJ and Schrader JW, Steel factor-induced tyrosine phosphorylation in murine mast cells. *J Immunol* **149:** 2772–2783, 1992.
19. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685, 1970.
20. Lee SL, Stevens JS, Wang WW and Lanzillo JJ, A liquid gelatin blocking reagent for western blotting with chemiluminescent detection. *Biotechniques* **17:** 60–62, 1994.
21. Gibbs JB, Pompliano DL, Mosser SD, Rands E, Lingham RB, Singh SB, Scolnick EM, Kohl NE and Oliff A, Selective inhibition of farnesyl-protein transferase blocks ras processing *in vivo*. *J Biol Chem* **268:** 7617–7620, 1993.
22. O'Donnell VB, Tew DG, Jones OTG and England PJ, Studies on the inhibitory mechanism of iodonium compounds with special reference to neutrophil NADPH oxidase. *Biochem J* **290:** 41–49, 1993.
23. Ellis C, Moran M, McCormick F and Pawson T, Phosphorylation of GAP and GAP-associated proteins by transforming and mitogenic tyrosine kinase. *Nature* **343:** 377–381, 1990.
24. Murrell GA, Francis MJ and Bromley L, Modulation of fibroblast proliferation by oxygen free radicals. *Biochem J* **265:** 659–665, 1990.
25. Rao GN and Berk BC, Active oxygen species stimulate vascular smooth muscle cell growth and protooncogene expression. *Circ Res* **70:** 593–599, 1992.
26. Bors W, Heller W, Michel C and Saran M, Flavonoids as antioxidants: Determination of radical-scavenging efficiencies. *Methods Enzymol* **186:** 343–355, 1990.
27. Chen Y, Zheng R, Jia Z and Ju Y, Flavonoids as superoxide scavengers and antioxidants. *Free Radic Biol Med* **9:** 19–21, 1990.
28. Robak J and Gryglewski RJ, Flavonoids are scavengers of superoxide anions. *Biochem Pharmacol* **37:** 837–841, 1988.
29. Seuwen K, Magnaldo I and Pouysségur J, Serotonin stimulates DNA synthesis in fibroblasts acting through 5-HT_{1B} receptors coupled to a G_i-protein. *Nature* **335:** 254–256, 1988.