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Antioxidants inhibit endothelin-1 (1–31)-induced proliferation of vascular smooth muscle cells via the inhibition of mitogen-activated protein (MAP) kinase and activator protein-1 (AP-1)

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Abstract

We previously found that human chymase cleaves big endothelins (ETs) at the Tyr^{31}–Gly^{32} bond and produces 31-amino acid ETs (1–31), without any further degradation products. In the present study, we investigated the effects of various antioxidants on the ET-1 (1–31)-induced change in intracellular signaling and proliferation of cultured rat aortic smooth muscle cells (RASMC). ET-1 (1–31) stimulated rapid and significant activation of the mitogen-activated protein (MAP) kinase family, i.e. extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun NH_2-terminal kinase (JNK), and p38 MAPK, in RASMC to an extent similar to that of ET-1. All of the antioxidants examined, i.e. *N*-acetyl-L-cysteine (NAC), diphenyleneiodonium chloride (DPI), and L-(+)-ascorbic acid (ascorbic acid), inhibited both ET-1 (1–31)- and ET-1-induced JNK and p38 MAPK activation but not ERK1/2 activation. Electron paramagnetic resonance (EPR) spectroscopy measurements revealed that NAC, DPI, and ascorbic acid inhibited xanthine oxidase-induced superoxide ($O_2^{\bullet -}$) generation in a cell-free system. ET-1 (1–31) in addition to ET-1 increased the generation of cellular reactive oxygen species (ROS) in RASMC. ET-1 (1–31)- and ET-1-induced cellular ROS generation was inhibited similarly by NAC, DPI, and ascorbic acid in RASMC. Gel-mobility shift analysis showed that ET-1 (1–31) and ET-1 caused an increase in activator protein-1 (AP-1)–DNA binding activity in RASMC that was inhibited by the above three antioxidants. ET-1 (1–31) increased [^{3}H]thymidine incorporation into cells to an extent similar to that of ET-1. This ET-1 (1–31)-induced increase in [^{3}H]thymidine incorporation was also inhibited by NAC and DPI, but not by ascorbic acid. These results suggest that antioxidants inhibit ET-1 (1–31)-induced RASMC proliferation by inhibiting ROS generation within the cells. The underlying mechanisms of the inhibition of cellular proliferation by antioxidants may be explained, in part, by the inhibition of JNK activation and the resultant inhibition of AP-1–DNA binding.

Keywords: Endothelin-1 (1–31); Human chymase; Mitogen-activated protein kinases; Reactive oxygen species; Antioxidants; Rat aortic smooth muscle cell

1. Introduction

It has been reported that the proliferation of VSMC is mediated by ROS, which are derived, at least in part, from a flavin-containing membrane-associated NADH/NADPH oxidase [1,2]. NADH/NADPH oxidase has also been reported to be the major source of ROS in several animal models of vascular disease [3,4]. Ang II was demonstrated to be a potent inducer of ROS through the activation of NADH/NADPH oxidase in VSMC [1]. In addition, ROS generation in response to various external stimuli has been shown to relate to the activation of MAP kinase [5] and AP-1 [6]. In a previous study, we demonstrated that antioxidants inhibit Ang II-induced JNK and p38 MAP kinase activation in RASMC [7], suggesting an important role of ROS in VSMC. A growing body of evidence suggests that ET-1 plays a role in the pathogenesis of several cardiovascular diseases associated with VSMC proliferation [8,9] and also induces ROS generation in different cell types

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Abbreviations: Ang II, angiotensin II; AP-1, activator protein-1; DMEM, Dulbecco's Modified Eagle's Medium; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DPI, diphenyleneiodonium chloride; DTPA, diethylenetriaminopentaacetic acid; ERK1/2, extracellular signal-regulated kinase 1/2; ET-1, endothelin-1; HPX, hypoxanthine; JNK, c-Jun NH_2-terminal kinase; MAP, mitogen-activated protein; p38 MAPK, p38 mitogen-activated protein kinase; NAC, *N*-acetyl-L-cysteine; PMSF, phenylmethylsulfonyl fluoride; RASMC, rat aortic smooth muscle cell(s); ROS, reactive oxygen species; SOD, superoxide dismutase; VSMC, vascular smooth muscle cell(s); XOD, xanthine oxidase.

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[10–12]. ET-1 like Ang II has been shown to induce ROS-dependent activation of MAP kinase in VSMC [13]. Since we previously reported that human chymase cleaves big endothelins (ETs) at the Tyr^{31}–Gly^{32} bond and produces 31-amino acid ETs (1–31), without any further degradation products [14], and that ET-1 (1–31) acts as an alternative mitogen for VSMC, induces human VSMC proliferation, and is almost equipotent to ET-1 [15], we hypothesized that ROS may also mediate ET-1 (1–31)-stimulated intracellular signaling cascades involving VSMC proliferation.

In the present study, we investigated the effects of the antioxidants NAC, DPI, and L-(+)-ascorbic acid (ascorbic acid) on ET-1 (1–31)- as well as ET-1-induced MAP kinase activation, i.e. ERK1/2, JNK, and p38 MAPK, in cultured RASMC. In addition, we directly measured the scavenging effects of these antioxidants on ROS in a cell-free system and characterized the ROS generation by ET-1 (1–31) and ET-1 in RASMC, using EPR spectroscopy. Finally, the effects of antioxidants on AP-1 activation, and the increase in DNA synthesis evoked by ET-1 (1–31) and ET-1 were examined.

2. Materials and methods

2.1. Chemicals

Human ET-1 (1–31) and human ET-1 were purchased from the Peptide Institute, Inc. NAC, DPI, SB 203580 (4-[4-fluorophenyl]-2-[4-methylsulfinyl-phenyl]-5-[4-pyridyl]-1H-imidazole), DTPA, XOD (from buttermilk), and HPX were obtained from the Sigma Chemical Co. L-(+)-Ascorbic acid was from Wako Pure Chemical Industries, Ltd. Phospho-ERK 1/2 antibody (Thr^{202}/Tyr^{204}), phospho-p38 MAPK antibody (Thr^{180}/Tyr^{182}), and an SAPK/JNK assay kit were purchased from New England Biolabs, Inc. DMPO was purchased from Labotech. All other chemicals were of reagent grade, were obtained from commercial sources, and were used without further purification.

2.2. Culture of RASMC

Treatment of the rats was based on the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication No. 85-23, revised 1985). The thoracic aortae of male Sprague–Dawley rats were excised rapidly and immersed in DMEM containing penicillin (100 U/mL)/streptomycin (100 μg/mL). The aortae were freed of connective tissue and adherent fat. The isolated arteries were cut open, and the endothelium was removed by gently rubbing off the intimal surface with sharp scissors. Denuded aortae were cut into ~3-mm cubes and placed intimal-face-down into three 35-mm culture dishes (IWAKI). DMEM containing 10% fetal bovine serum and penicillin/streptomycin was gently added to the dishes to cover the tissues without disturbance to the orientation of the explants. VSMC were allowed to grow out from the tissue (7–10 days), and the tissues were removed (using sterilized fine forceps); the VSMC outgrowths were washed with culture medium. After reaching confluence in three 35-mm dishes, cells were harvested by brief trypsinization and grown in T-75 flasks (IWAKI) (passage 1). Early subcultured cells (from passages 2–5) were used in all experiments. The purity of the VSMC was estimated to be >90% by morphology and by the expression of myosin as described previously [16]. Cell viability was >98% as determined by exclusion of 0.2% trypan blue.

2.3. Preparation of cell lysates for MAP kinase activity assay

RASMC were seeded ($1–2 \times 10^4$ cells cm^{-2}) and grown to subconfluence in 60-mm culture dishes (IWAKI) in DMEM–10% fetal bovine serum. Cells were made quiescent for 48 hr in serum-free DMEM and preincubated with or without antioxidants for various times (30 min to 2 hr) followed by stimulation with ET-1 (1–31) (100 nM) or ET-1 (100 nM) at the indicated time points. The incubation medium was discarded, and cell monolayers were washed once with cold PBS containing sodium orthovanadate (1 mM). Cells were lysed (30–60 min at 4°) with cell lysis buffer consisting of 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 μg/mL of leupeptin, and 1 mM PMSF. After incubation, lysed cells were sonicated (Handy Sonic UR-20 P; Tomy Seiko Co, Ltd.) on ice for 1 min, transferred to microcentrifuge tubes, and centrifuged at 16,000 *g* for 20 min at 4°. The protein concentrations of the supernatants were measured with a protein assay kit (Pierce) and stored at −80° until assayed for MAP kinase activity.

2.4. Measurements of ERK1/2 and p38 MAPK activities in RASMC

Previously, we measured each MAP kinase activity using an in-gel kinase assay with specific substrates. However, we found that the activities of ERK1/2 and p38 MAPK, as measured by the in-gel kinase assay, and their phosphorylation states, as assayed by immunoblotting, were highly correlated ($R^2 = 0.90$) in RASMC (unpublished data). Therefore, we used immunoblotting for the detection of phospho-ERK1/2 and phospho-p38 MAPK to evaluate ERK1/2 and p38 MAPK activation, as described previously [17]. For immunoblot analysis, cell lysates were subjected to SDS–PAGE, and proteins were transferred to nitrocellulose membranes (Hybond™-ECL; Amersham Pharmacia Biotech), as described previously [17]. The membranes were blocked for 1 hr at room temperature with a commercial blocking buffer from

Amersham Pharmacia Biotech. The blots were then incubated for 12 hr with anti-phosphospecific ERK1/2 or p38 MAPK antibodies (New England Biolabs), followed by incubation for 1 hr with a secondary antibody (conjugated horseradish peroxidase). Immunoreactive bands were visualized using enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech) and were quantified by densitometry in the linear range of film exposure using a UMAX Astra 2200 scanner (UMAX Technologies) and NIH image 1.60 software.

2.5. JNK activity assay

JNK activity was measured with a commercially available kit based on the phosphorylation of recombinant c-Jun (New England Biolabs). After treatment, cells were rinsed twice with ice-cold PBS, scraped off the plates into lysis buffer (included in the kit), and sonicated three times on ice. After removing the cell debris by centrifugation (16,000 *g*, 20 min, 4°), the protein content in the supernatant was measured using a protein assay kit (Pierce). Equal amounts of protein (300 μg) were then immunoprecipitated with c-Jun (1-89) fusion protein beads overnight. After washing the beads, kinase assays were performed according to the instructions of the manufacturer. Beads were loaded on a 10% SDS–polyacrylamide gel, and immunoblotting was performed with an antibody against phosphospecific c-Jun [17].

2.6. Measurements of the superoxide scavenging activity of antioxidants by the EPR-spin trapping method

The $O_2^{\bullet -}$ scavenging activities of NAC, DPI, and ascorbic acid were studied by the EPR-spin trapping method. Several concentrations of NAC, DPI, and ascorbic acid were introduced into the reaction mixture containing 2 mM HPX, 0.272 U/mL of XOD, and 10% DMPO in 0.1 M HEPES buffer (pH 7.4), and then kept at 37°. One minute after the addition of XOD, samples were transferred into a quartz flat cell, which was, in turn, placed in the cavity of the EPR spectrometer to measure the DMPO/OO$^{\bullet}$ signal. Spectra were stored on an IBM/PC computer with software (ESPRIT 432; JEOL Co, Ltd.) for analysis. Detailed EPR conditions are in the figure legends.

2.7. Measurements of cellular ROS generation in RASMC

To estimate ROS production, we adopted a slight modification of the EPR-spin trapping method described previously [18]. RASMC were seeded (1–2 × 10^4 cells cm^{-2}) and grown to subconfluence in 60-mm culture dishes (IWAKI) in DMEM–10% fetal bovine serum. Cells were made quiescent for 48 hr in serum-free DMEM and preincubated with or without antioxidants for various times (30 min to 2 hr). The incubation medium was discarded, and cells were washed twice with PBS containing 0.1% glucose. PBS containing 0.1 mM DTPA was added and stimulated with ET-1 (1–31) (100 nM) or ET-1 (100 nM) plus 100 mM DMPO in the presence or absence of antioxidants for 5 min at 37°. Cells were harvested with a sterile rubber scraper and transferred into capillary tubes. Then, the DMPO/$^{\bullet}$OH spin adduct was measured to determine cellular ROS generation, using EPR spectroscopy.

2.8. EPR spectroscopy

EPR spectra were recorded in capillary tubes at room temperature with a JES-TE 300 spectrometer (JEOL Co, Ltd.) operating at X-band with 100-kHz modulation frequency and an ES-UCX2 cavity. Spectral simulations were performed using a simulation program, Winsim [19]. Quantitation of spectra with a single free radical signal was performed by comparison of the double integral of the observed signal with that of the 3rd EPR spectrum of Mn^{2+}.

2.9. Gel mobility shift assay

For the gel mobility shift assay, nuclear protein extracts were prepared from RASMC in 60-mm dishes after preincubation with or without antioxidants or SB 203580 for various times (30 min to 2 hr) followed by stimulation with ET-1 (1–31) (100 nM) or ET-1 (100 nM) at the indicated time points. The samples were homogenized in 0.4 mL of 20 mM HEPES (pH 7.9) containing 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1.5 mM $MgCl_2$, 20% glycerol, 10 mM NaF, 1 mM Na_3VO_4, 0.2 mM dithiothreitol (DTT), 20 mM β-glycerophosphate, 0.5 mM PMSF, 60 μg/mL of aprotinin, and 2 μg/mL of leupeptin, incubated on ice for 15 min, and centrifuged at 15,000 *g* for 10 min at 4°. The resulting supernatants were assayed for protein concentration and stored at −80° until used. The procedure for the gel mobility shift assay has been described previously [20,21]. In brief, the gel mobility shift assay of RASMC nuclear AP-1 binding activity was performed with an oligonucleotide probe containing the AP-1 binding sequence (5′-CGCTTGATGACTCAGCCGGAA-3′) [22]. The probe was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase, and purified by chromatography on a Bio-Spin column (Bio-Rad, Alfred Nobel Drive Hercules). For the DNA–protein binding reaction, the samples of RASMC nuclear protein extract (10 μg protein) were incubated with 10 fmol of a ^{32}P-labeled oligonucleotide containing the consensus AP-1 binding site at room temperature for 20 min, in 20 μL of binding buffer consisting of 20 mM HEPES (pH 7.9), 0.2 mM EDTA, 0.2 mM EGTA, 80 mM NaCl, 0.3 mM $MgCl_2$, 1 mM DTT, 0.2 mM PMSF, 6% glycerol, and 2 μg of polydeoxyinosinic-deoxycytidylic acid (poly[dI-dC]; Pharmacia Biotech) as a nonspecific competitor. The

DNA–protein complexes were separated from the free DNA probe by electrophoresis on 4% nondenaturing polyacrylamide gels in 6.7 mM Tris–HCl (pH 7.5), 3.3 mM sodium acetate, 0.1 mM EDTA, and 2.5% glycerol. Gels were run at 200 V at 4° for 3 hr, dried, subjected to autoradiography, and analyzed with a bioimaging analyzer (BAS-2000 Fuji Film).

To demonstrate the specificity of DNA–protein binding, binding reactions were performed as described above, in the presence of a 100-fold molar excess of a non-labeled AP-1 consensus oligonucleotide competitor, and followed by electrophoresis. Furthermore, to examine the possible contribution of c-Jun to specific AP-1 binding activity, supershift assays were performed with rabbit anti-c-Jun IgG raised against amino acids 247–263 of c-Jun (Santa Cruz Biotechnology). Anti-c-Jun IgG (each 1 μg) was added to the samples after the initial binding reaction between the arterial protein extracts and the ^{32}P-labeled consensus AP-1 oligonucleotide. The reaction was allowed to proceed at room temperature for 1 hr, and then the samples were subjected to electrophoresis, as described above.

2.10. Determination of [^{3}H]thymidine incorporation

Subconfluent RASMC in 12-well culture plates were made quiescent by placing them in serum-free DMEM for 2 days. They were then stimulated with ET-1 (1–31) (100 nM) or ET-1 (100 nM) in the presence or absence of antioxidants or SB 203580 for 24 hr. The stimulated cells were pulsed with 1 μCi/mL of [^{3}H]thymidine during the last 8 hr of culture. Cells were washed once with PBS, and twice with ice-cold 5% trichloroacetic acid (TCA) to remove the unincorporated [^{3}H]thymidine, then solubilized in 100 μL of 0.25 N NaOH in 0.1% SDS, and neutralized. Aliquots of samples were added to 10 mL of scintillation fluid, and radioactivity was assessed (Aloka 703).

2.11. Statistical analysis

Values are reported as the means ± SD from experiments done in quintuplicate. Two-way ANOVA was used to determine the significance among groups, after which the modified *t*-test with the Bonferroni's *post hoc* test was used for comparison between individual groups. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Time course for the activation of ERK1/2, JNK, and p38 MAPK by ET-1 (1–31) or ET-1 in RASMC

To evaluate the relative magnitude of MAP kinase activation by ET-1 (1–31) or ET-1, growth-arrested RASMC were exposed to ET-1 (1–31) (100 nM) or ET-1 (100 nM). ERK1/2, JNK, and p38 MAPK activities in the cell lysates were determined as described in "Materials and methods." ET-1 (1–31) activated ERK1/2 and JNK within 5 min and peaked at 15 min (5.64- and 3.16-fold, respectively). p38 MAPK was activated within 5 min and peaked at 10 min (3.25-fold) after stimulation by ET-1 (1–31) (Fig. 1A). As shown in Fig. 1C, ERK1/2 was activated and peaked within 5 min (4.4-fold) after stimulation by ET-1. JNK and p38 MAPK were activated within 5 min and peaked at 10 min (2.3- and 2.34-fold, respectively) after stimulation by ET-1. No alterations in the amounts or total expression of c-Jun fusion protein, ERK1/2, and p38 MAPK were observed by the stimulation (Fig. 1A and C). These findings are summarized in panels B and D of Fig. 1.

3.2. Concentration–response curve for the activation of ERK 1/2, JNK, and p38 MAPK by ET-1 (1–31) or ET-1 in RASMC

We also determined the concentration-dependence for MAP kinase activation by the indicated concentrations of ET-1 (1–31) or ET-1 in RASMC for the following times: ERK1/2 and JNK, 15 min; and p38 MAPK, 10 min for ET-1 (1–31) stimulation and ERK1/2, 5 min; and JNK and p38 MAPK, 10 min for ET-1 stimulation. ET-1 (1–31)- or ET-1-induced ERK1/2, JNK, and p38 MAPK activations increased in a concentration-dependent manner (from 10^{-10} to 10^{-7} M). ET-1 (1–31)- or ET-1-stimulated ERK1/2, JNK, and p38 MAPK activation was maximal at 100 nM. The magnitude of JNK and p38 MAPK activation was smaller than that of ERK1/2 at high concentrations of ET-1 (1–31) or ET-1 (Fig. 2A–D). No alterations in the amounts or total expression of c-Jun fusion protein, ERK1/2, and p38 MAPK were observed by the stimulation (Fig. 2A and C).

3.3. Effects of the antioxidants NAC, DPI, and ascorbic acid on the EPR signal of the DMPO/OO$^\bullet$ spin adduct

To clarify the $O_2^{\bullet-}$ scavenging activity of antioxidants in the cell-free system, we conducted the EPR-spin trapping method with DMPO to measure the production of the DMPO/OO$^\bullet$ spin adduct by XOD and the HPX system. When 0.272 U/mL of XOD was added to the reaction mixture containing 2 mM HPX and 2.7 M DMPO in Chelex-treated 0.1 M HEPES buffer (pH 7.4), an apparent DMPO/OO$^\bullet$ spin adduct was observed (Fig. 3A, C, and E depicted as "Control") with hyperfine splitting constants of $a^N = 14.15$ and $a^H = 11.37$ G as reported previously [23]. Next, we studied the effects of NAC (from 10^{-5} to 10^{-1} M), DPI (10^{-7} to 10^{-3} M), and ascorbic acid (from 10^{-6} to 10^{-2} M) on the formation of the DMPO/OO$^\bullet$ spin adduct from XOD and the HPX system. As shown in Fig. 3, these antioxidants decreased the EPR signals of the

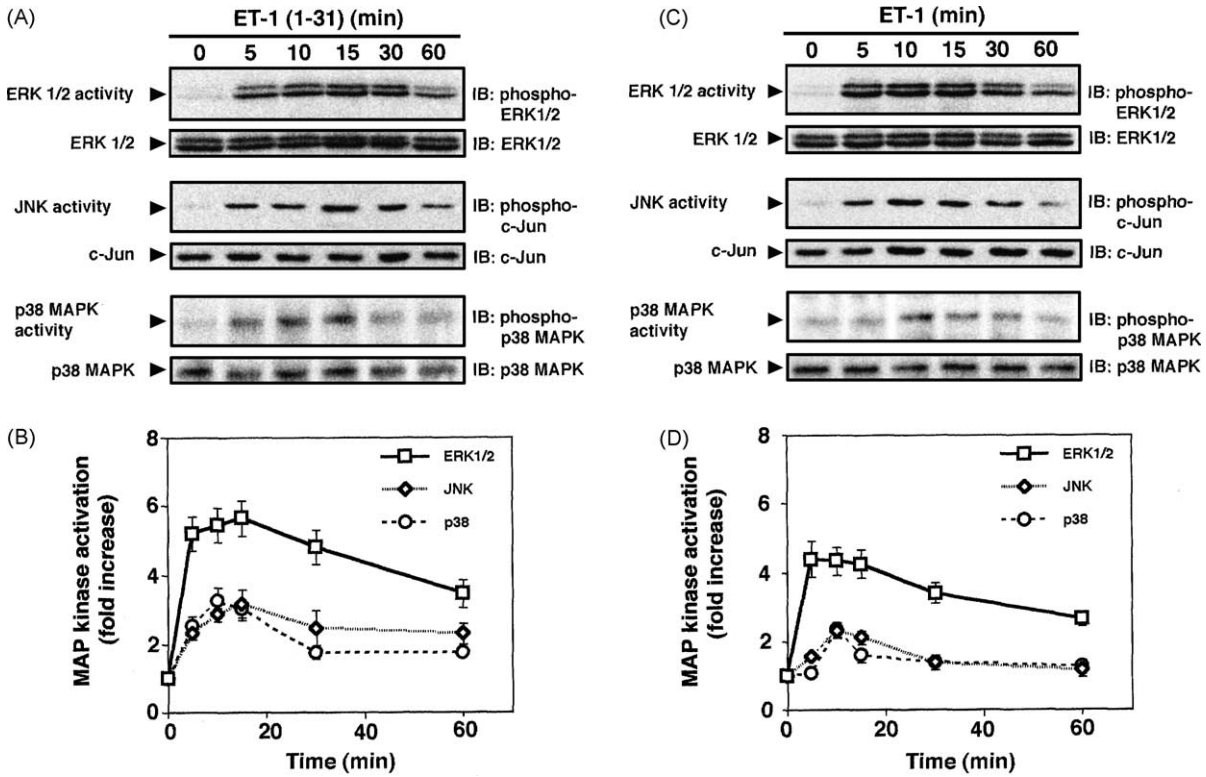

Fig. 1. Time course of ET-1 (1–31)- or ET-1-induced ERK1/2, JNK, and p38 MAPK activation in RASMC. (A and C) Representative blots. (B and D) Densitometric analyses of ERK1/2, JNK, and p38 MAPK activation. Values (means ± SD, N = 5) were normalized by arbitrarily setting the densitometry of control cells (time = 0) to 1.0.

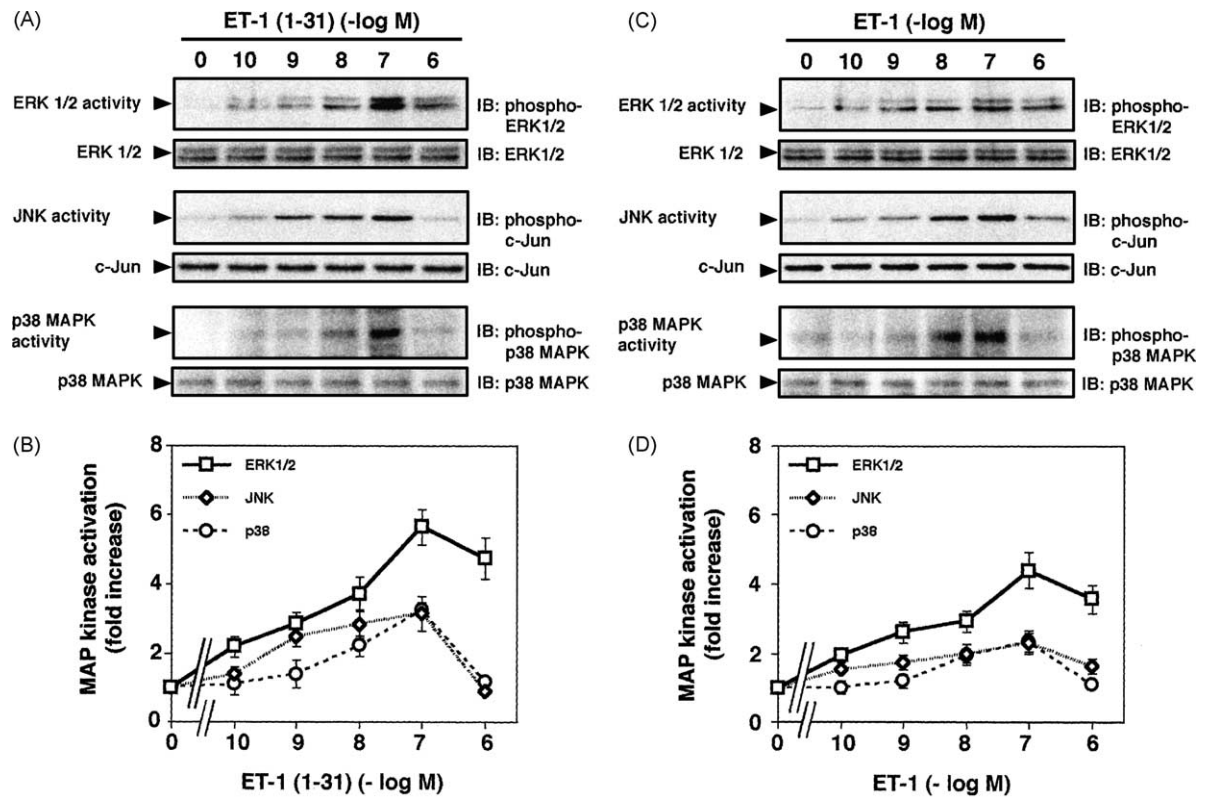

Fig. 2. Concentration–response curves of ET-1 (1–31)- or ET-1-induced ERK1/2, JNK, and p38 MAPK activation in RASMC. (A and C) Representative blots. (B and D) Densitometric analyses of ERK1/2, JNK, and p38 MAPK activation. Values (means ± SD, N = 5) were normalized by arbitrarily setting the densitometry of control cells (without agonists) to 1.0.

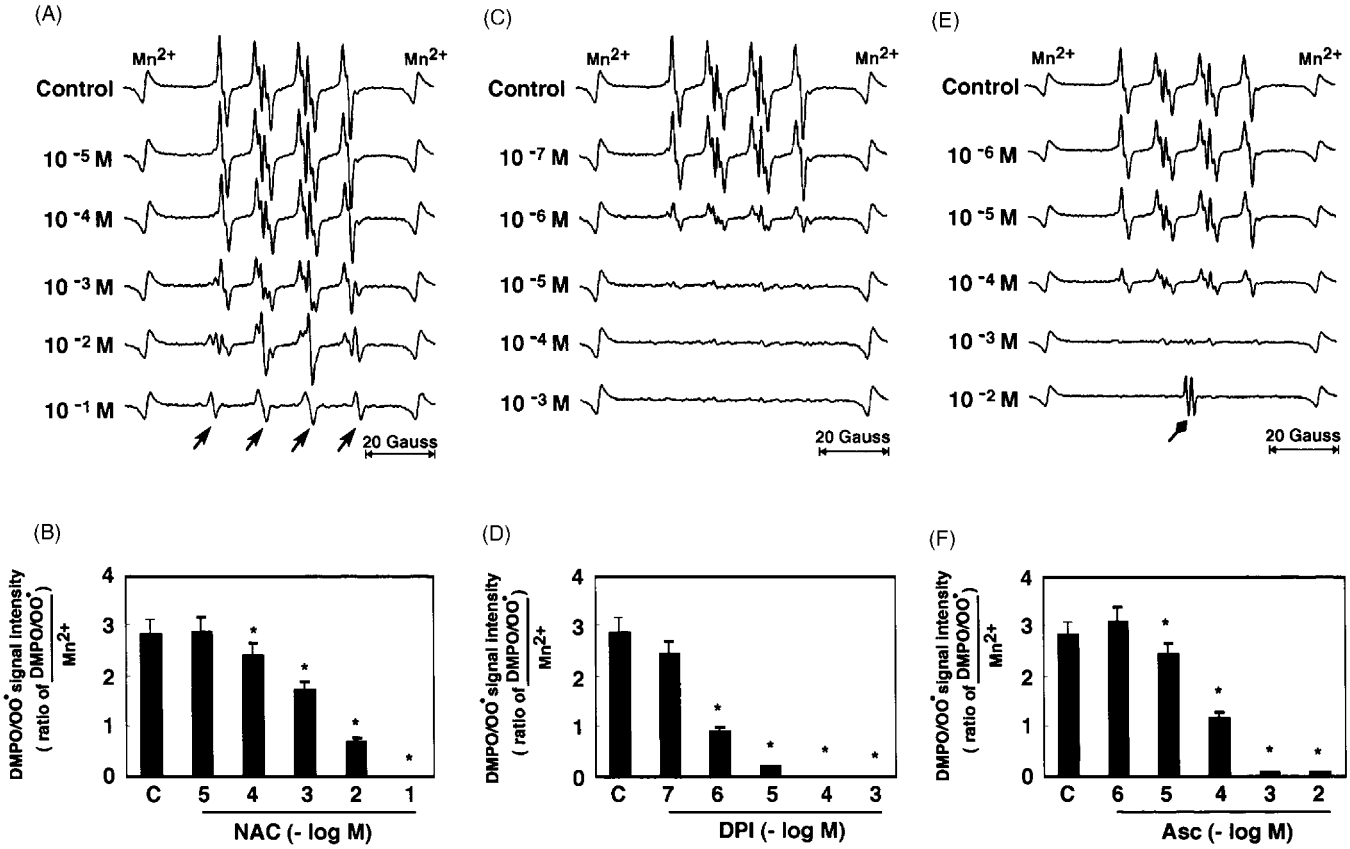

Fig. 3. Effects of antioxidants on the EPR signal of the DMPO/OO• spin adduct in a cell-free system. (A, C, and E) Representative EPR signals of the DMPO/OO• spin adduct. As shown in the bottom EPR spectra of panels A and E, the closed arrows and the diamond arrow indicate EPR signals of the DMPO/S• spin adduct and the ascorbate radical, respectively. (B, D, and F) Values (means ± SD, N = 5) are expressed as the ratio of the EPR signal intensity between the first peak of the DMPO/OO• spin adduct and the left-sided peak of the Mn^{2+}. Key: (*) significantly different from the controls ($P < 0.05$).

DMPO/OO• spin adduct in a concentration-dependent manner. In the case of NAC and ascorbic acid, EPR signals of the DMPO/S• spin adduct ($a^N = 15.12$, $a^H = 16.37$ G) [24] and ascorbate radical ($a^H = 1.79$ G) [25] appeared concomitant with the decrease in the DMPO/OO• spin adduct, respectively.

3.4. Effects of the antioxidants NAC, DPI, and ascorbic acid on the EPR signal of the DMPO/•OH spin adduct induced by ET-1 (1–31) or ET-1 in RASMC

To evaluate cellular ROS generation, we directly measured the EPR signal of the DMPO/•OH spin adduct in RASMC using the EPR-spin trapping method. ET-1 (1–31) (100 nM) or ET-1 (100 nM) caused a significant increase in the EPR signal of the DMPO/•OH spin adduct in RASMC observed with hyperfine splitting constants of $a^N = 15.05$ and $a^H = 14.65$ G [26]. We next determined the effects of the antioxidants on the ET-1 (1–31)- or ET-1-stimulated increase in EPR signal of the DMPO/•OH spin adduct. The cells were pretreated with NAC (10 mM) or ascorbic acid (100 μM) for 30 min, and DPI (10 μM) for 2 hr before the stimulation of ET-1 (1–31) (100 nM) or ET-1 (100 nM) for a 5-min incubation period with 0.1 M DMPO. EPR signals of the DMPO/•OH spin adducts were decreased significantly by the addition of NAC (10 mM), DPI (10 μM), and ascorbic acid (100 μM) (Fig. 4).

3.5. Effects of the antioxidants NAC, DPI, and ascorbic acid on the activation of ERK1/2, JNK, and p38 MAPK by ET-1 (1–31) or ET-1 in RASMC

To clarify the role of ROS in ET-1 (1–31)- or ET-1-induced MAP kinase activation, we studied the effects of different antioxidants on ET-1 (1–31)- or ET-1-induced ERK1/2, JNK, and p38 MAPK activation. The cells were pretreated with NAC (10 mM) or ascorbic acid (100 μM) for 30 min, and with DPI (10 μM) for 2 hr before the stimulation of ET-1 (1–31) (100 nM) or ET-1 (100 nM) for a 10- to 15-min incubation period. ET-1 (1–31)- or ET-1-stimulated ERK1/2 activation was not affected by any of the antioxidants examined, whereas JNK and p38 MAPK activation were both inhibited by each antioxidant (Fig. 5A–D). We have already confirmed that all of the antioxidants examined had no effect on basal MAP kinase activity in VSMC [7].

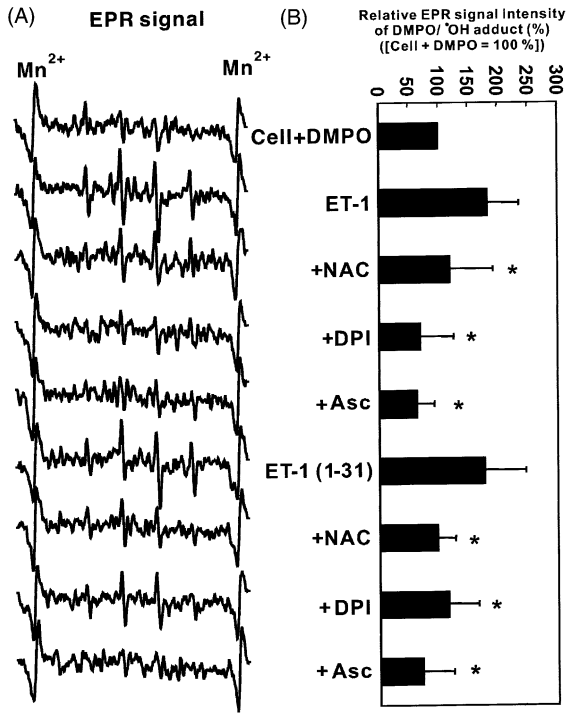

Fig. 4. Effects of antioxidants on the ET-1 (1–31)- or the ET-1-induced EPR signal of the DMPO/•OH spin adduct in RASMC. (A) Representative EPR signals of the DMPO/•OH spin adduct. (B) Values (means ± SD, N = 5) are expressed as a percentage of controls (without agonists) to 100%. Key: (*) significantly different from the values of ET-1 (1–31)- or ET-1-induced EPR signals of the DMPO/•OH spin adduct ($P < 0.05$).

3.6. Effects of antioxidants and SB 203580 on the increase in AP-1–DNA binding activity by ET-1 (1–31) or ET-1 in RASMC

Since ROS generation in response to various external stimuli has been shown to relate to the activation of AP-1–DNA binding, we examined the effects of different antioxidants on ET-1 (1–31)- or ET-1-induced AP-1–DNA binding activities. The incubation of a consensus AP-1 oligonucleotide with RASMC nuclear extracts resulted in the formation of a broad band of AP-1 complexes. This broad band was found to have specific binding for AP-1, because the addition of 100-fold molar excess of an unlabeled AP-1 consensus oligonucleotide resulted in a complete competitive inhibition of the formation of AP-1 complexes. Furthermore, the addition of anti-c-Jun antibody to the binding reaction induced supershifted complexes (Fig. 6A, C, and E). ET-1 (1–31) (100 nM) or ET-1 (100 nM) treatment for 2 hr increased DNA binding activities in the nuclear extracts of RASMC by 2.3- and 2.5-fold, respectively, compared with the control. These AP-1–DNA binding activities induced by ET-1 (1–31) or ET-1 were inhibited significantly by pretreatment with NAC (10 mM) or ascorbic acid (100 μM) for 30 min, and with DPI (10 μM) for 2 hr before stimulation with ET-1 (1–31) (100 nM) or ET-1 (100 nM) for a 2-hr period (Fig. 6A–D). We also examined whether p38 MAPK is involved in AP-1–DNA binding activities by using SB 203580, a specific p38 MAPK inhibitor. However, SB 203580 showed no significant effect on the ET-1 (1–31)- or ET-1-induced formation of a broad band of AP-1 complexes (Fig. 6E and F).

3.7. Effects of antioxidants and SB 203580 on DNA synthesis stimulated by ET-1 (1–31) or ET-1 in RASMC

To clarify the role of ROS in DNA synthesis in RASMC, we examined the effects of different antioxidants on ET-1 (1–31)- or ET-1-induced increases in [^{3}H]thymidine incorporation into RASMC. Cells were growth arrested and stimulated with ET-1 (1–31) (100 nM) or ET-1 (100 nM) in the presence or absence of NAC (10 mM), ascorbic acid (100 μM), DPI (10 μM), and SB 203580 (10 μM) for a 24-hr incubation period. ET-1 (1–31) or ET-1 similarly increased [^{3}H]thymidine incorporation into RASMC. These effects were inhibited significantly by NAC and DPI. However, ascorbic acid itself caused an increase in [^{3}H]thymidine incorporation into RASMC and failed to inhibit either ET-1 (1–31)- or ET-1-induced increases in [^{3}H]thymidine incorporation into RASMC. In this study, we also demonstrated that SB 203580 had no significant effect on ET-1 (1–31)- or ET-1-induced increase in [^{3}H]thymidine incorporation (Fig. 7).

4. Discussion

Human mast cell chymase selectively cleaves big ETs at the Tyr^{31}–Gly^{32} bond to produce novel trachea-constricting 31-amino acid endothelins, ETs (1–31), without any further degradation products [14]. ET-1 (1–31) has been known to act as an alternative mitogen for VSMC, to cause HASMC proliferation, and to be almost equipotent to ET-1 [15]. In the present study, we demonstrated that ET-1 (1–31) caused a rapid and significant activation of MAP kinase members ERK1/2, JNK, and p38 MAPK in a concentration-dependent manner (10^{-10} to 10^{-7} M) in RASMC to an extent similar to that of ET-1 (Figs. 1 and 2). These results are consistent with those of a previous study that reported that ET-1 (1–31) stimulated the activation of ERK1/2 in human VSMC as well as in human mesangial cells to an extent similar to that of ET-1 [15,27]. We also reported that ET-1 (1–31) activates p38 MAPK in human mensangial cells [28].

The NADH/NADPH oxidase enzyme has been known to produce the superoxide anion radical ($O_2^{\bullet -}$) in response to extracellular stimuli such as Ang II, endothelin-1, growth factors, and cytokines [1–4]. It has also been reported that XOD can contribute to ROS generation within cells [29]. In the present study, we first examined the radical scavenging effect of the antioxidants NAC, DPI, and ascorbic acid in a cell-free system using EPR spectroscopy. As shown

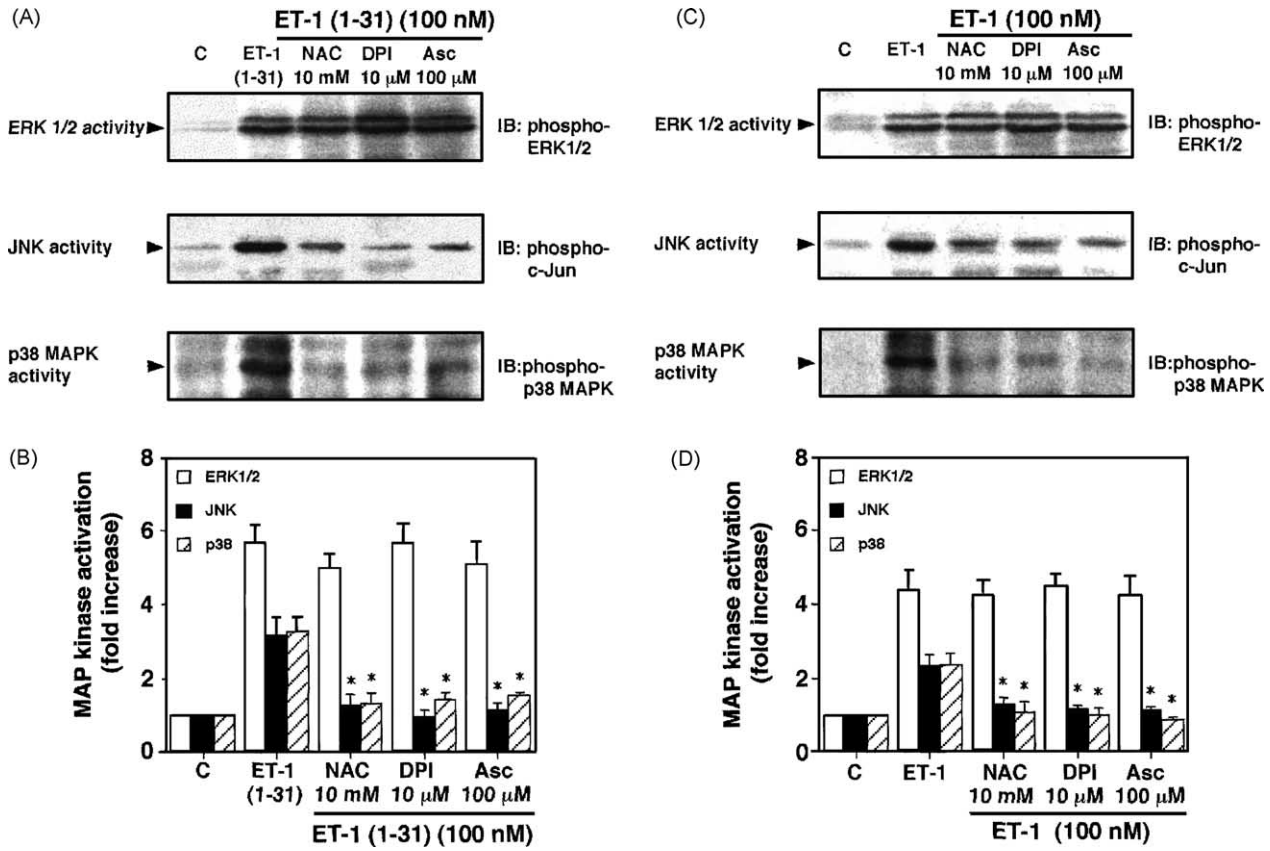

Fig. 5. Effects of antioxidants on ET-1 (1–31)- or ET-1-induced ERK1/2, JNK, and p38 MAPK activation in RASMC. (A and C) Representative blots. (B and D) Densitometric analyses of ERK1/2, JNK, and p38 MAPK activation. Values (means ± SD, N = 5) were normalized by arbitrarily setting the densitometry of control cells (without agonists) to 1.0. Key: (*) significantly different from the ET-1 (1–31)- or ET-1-induced MAP kinase activation ($P < 0.05$).

in Fig. 3, we directly measured the DMPO/OO$^{\bullet}$ signal intensity, which was derived originally from the $O_2^{\bullet-}$ generated via the reaction of HPX/XOD in an *in vitro* cell-free system. NAC, DPI, and ascorbic acid significantly inhibited HPX/XOD-induced $O_2^{\bullet-}$ generation in a concentration-dependent manner. Although DPI was originally found to be an inhibitor of NADH/NADPH oxidase, EPR measurement revealed that it also inhibits XOD, which has been reported to be involved in $O_2^{\bullet-}$ generation in cells [30]. We next demonstrated that the EPR signal of the DMPO/$^{\bullet}$OH spin adduct, indicating cellular ROS generation, in RASMC stimulated by ET-1 (1–31) or ET-1 was inhibited significantly by all of the antioxidants examined (Fig. 4). It has been reported that $O_2^{\bullet-}$ is readily converted to $^{\bullet}$OH via the Fenton reaction within the cells [31]. On the other hand, the EPR signal of the DMPO/$^{\bullet}$OH spin adduct can arise from the trapping of the $^{\bullet}$OH radical, or from the decay of the DMPO/OO$^{\bullet}$ spin adduct of the $O_2^{\bullet-}$ radical, which decomposes rapidly to form DMPO/$^{\bullet}$OH [32]. However, we could not determine whether or not the observed EPR signal of the DMPO/$^{\bullet}$OH spin adduct was originally derived from the $O_2^{\bullet-}$ radical, because SOD failed to decrease the observed EPR signal of the DMPO/$^{\bullet}$OH spin adduct under the present experimental conditions (data not shown). Since SODs are proteins with molecular weights ranging from 33,000 to 80,000, cellular penetration does not readily occur [33]. Thus, in the present study it is also likely that exogenous SOD may not have an effect on the intracellular $O_2^{\bullet-}$ radical. Taken together, the present study suggests that NAC, DPI, and ascorbic acid inhibited ET-1 (1–31)- or ET-1-induced cellular ROS generation probably through both radical scavenging effects on the $O_2^{\bullet-}$ or $^{\bullet}$OH radicals and inhibition effects on the $O_2^{\bullet-}$ radical generating enzyme system. These results are consistent with those of our previous study that showed that antioxidants prevented Ang II-induced radical generation evaluated by oxygen consumption as an index of ROS generation in RASMC [7].

ET-1 as well as Ang II can induce ROS-dependent activation of MAP kinase in VSMC [2,13]. It has also been reported that ET-1 causes ROS generation in cultured rat cardiomyocytes [12]. In addition, $p22^{phox}$, an essential component of NADH/NADPH oxidase, has been shown to be relevant to VSMC hypertrophy or atherosclerosis [34]. In the present study, we examined the effects of the antioxidants NAC, DPI, and ascorbic acid on ET-1 (1–31)- or ET-1-induced MAP kinase activation in RASMC. As shown in Fig. 5, JNK and p38 MAPK activation induced by ET-1 (1–31) or ET-1 was inhibited by all of the antioxidants tested, whereas ERK1/2 was not affected by them. These findings are consistent with those of Fei *et al.* [13], who demonstrated that JNK activation but not

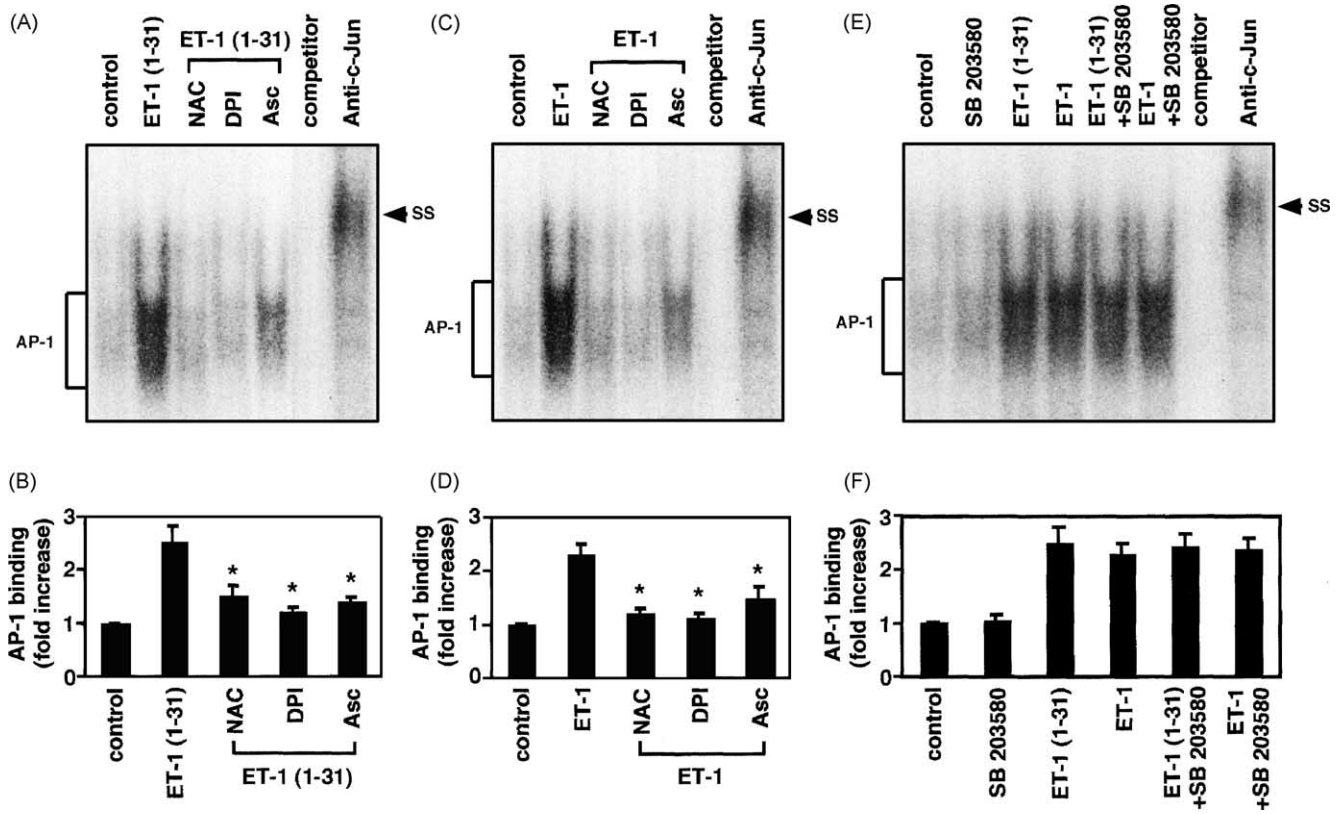

Fig. 6. Effects of antioxidants and SB 203580 on ET-1 (1–31)- or ET-1-induced AP-1–DNA binding activity in RASMC nuclear extract. (A, C, and E) A representative broad band of AP-1 complexes (AP-1) and a supershifted band (SS) are shown. (B, D, and F) Densitometric analyses of AP-1–DNA binding activities. Values (means ± SD, N = 5) were normalized by arbitrarily setting the densitometry of control cells (without agonists) to 1.0. Key: (*) significantly different from the ET-1 (1–31)- or ET-1-induced increase in AP-1–DNA binding activities ($P < 0.05$).

ERK1/2 activation by ET-1 was inhibited significantly by NAC and DPI. We have also shown that Ang II-induced JNK and p38 MAPK activation was inhibited by NAC, DPI, and ascorbic acid, although ERK1/2 activation was not inhibited [7]. These results suggest that JNK and p38 MAPK are sensitive to ROS but not ERK1/2. There may be other ROS-insensitive signaling pathways in ET-1 (1–31)- or ET-1-induced ERK1/2 activation.

MAP kinases such as ERK1/2 and JNK play a central role in the regulation of AP-1 expression and activation

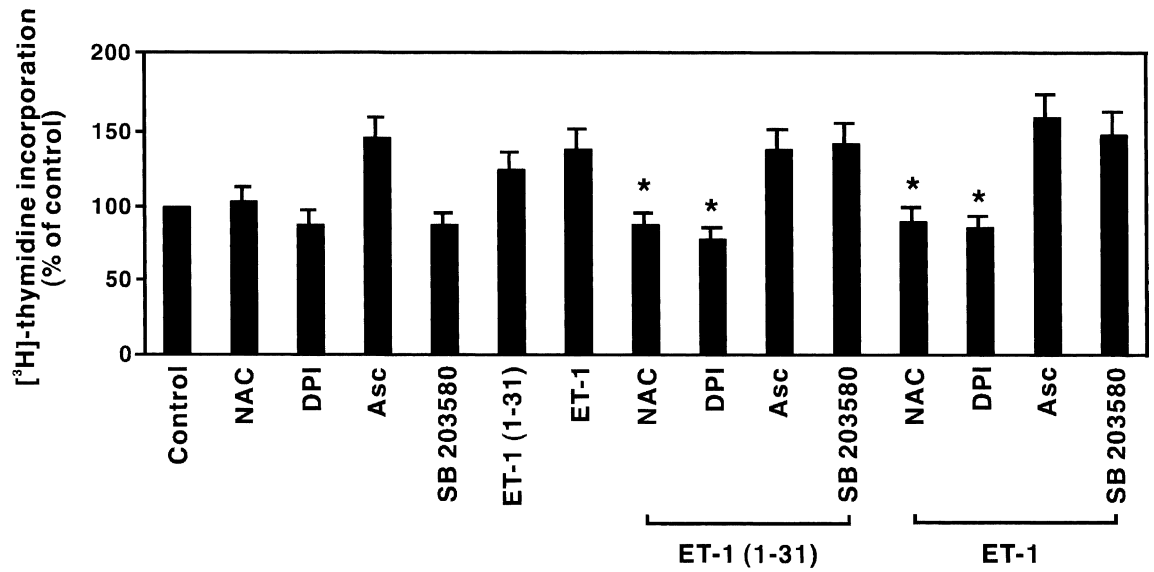

Fig. 7. Effects of antioxidants and SB 203580 on ET-1 (1–31)- or ET-1-induced increases in DNA synthesis in RASMC. Values (means ± SD, N = 5) are expressed as a percentage of controls (without agonists); 100% = 73,800 ± 7,500 cpm. Key: (*) significantly different from the ET-1 (1–31)- or ET-1-induced increase in [3H]thymidine incorporation ($P < 0.05$).

[35,36]. The JNK substrate c-Jun forms homodimers or heterodimers with c-Jun or c-Fos to form the AP-1 complex [37]. We previously reported that AP-1–DNA binding activity was increased significantly by ET-1 (1–31) as well as ET-1 in human VSMC [15]. ROS generation, in response to various external stimuli, has been shown to relate to the activation of MAP kinase [5] and AP-1 [6]. Therefore, in the present study, we examined whether antioxidants affect the ET-1 (1–31)- or ET-1-stimulated increase in AP-1–DNA binding activity in RASMC nuclear extract. As shown in panels A–D of Fig. 6, we provided evidence that antioxidants can inhibit ET-1 (1–31)- or ET-1-stimulated AP-1–DNA binding activity. It has also been reported that the ET-1-induced increase in AP-1 activity is radical dependent in RASMC [13]. In addition, the present study indicates that p38 MAPK may not be involved in ET-1 (1–31)- or ET-1-induced AP-1–DNA binding activity (Fig. 6E and F). Since ERK1/2 was insensitive to all of the antioxidants in the present study, ET-1 (1–31)- or ET-1-stimulated AP-1–DNA binding activity may be mediated through ROS-dependent JNK activation, although the present study provided no direct evidence of this.

It has been reported that ET-1 increases the incorporation of [^{3}H]thymidine into DNA and causes proliferation of VSMC [38]. We also reported that ET-1 (1–31) acts as an alternative mitogen for VSMC, induces cell proliferation, and is almost equipotent to ET-1 [15]. As shown in Fig. 7, the present results demonstrate that the ET-1 (1–31)-promoted DNA synthesis in RASMC was almost equivalent to that of ET-1. Recently, VSMC proliferation has been reported to be mediated by ROS, which are derived, at least in part, from a flavin-containing membrane-associated NADH/NADPH oxidase [1,2]. The present study demonstrates consistently that both NAC and DPI potently inhibited the ET-1 (1–31)- as well as the ET-1-induced increase in DNA synthesis in RASMC. In contrast, ascorbic acid alone caused an increase in DNA synthesis and failed to inhibit the ET-1 (1–31)- or ET-1-induced increase in DNA synthesis in RASMC. It is difficult to explain the effect of ascorbic acid on DNA synthesis; it has been reported that ascorbic acid plays a dual role as both an antioxidant and a prooxidant. Ascorbic acid may act as a prooxidant *in vitro* in the presence of metal ions [39,40], which may contribute one possible explanation for the increase in [^{3}H]thymidine incorporation into the cells in response to ascorbic acid. Further studies are needed to define the effect of ascorbic acid on VSMC proliferation. Although different studies have proposed that p38 MAPK mediates VSMC proliferation [41], hypertrophy [42], and apoptosis [43], it is unlikely that p38 MAPK is involved in VSMC proliferation because SB 203580, a specific p38 MAPK inhibitor, failed to inhibit the ET-1 (1–31)- or ET-1-induced increase in [^{3}H]thymidine incorporation under the present experimental conditions (Fig. 7).

Finally, we demonstrated in the present study that ET-1 (1–31)-induced RASMC proliferation is probably through ROS generation within the cells, which is inhibited by antioxidants. Furthermore, the ROS-sensitive intracellular signaling mechanism causing RASMC proliferation may be explained, in part, by the activation of JNK and the resultant activation of AP-1–DNA binding. Thus, the findings of the present study may enhance the important role of antioxidants in ROS-mediated ET-1 (1–31)-induced RASMC proliferation and suggest a clinical application of antioxidants in cardiovascular diseases.

Acknowledgments

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