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European Journal of Pharmacology 484 (2004) 119–125
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Antiproliferative mechanisms of raxofelast (IRFI-016) in H_2O_2-stimulated rat aortic smooth muscle cells

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Received 3 April 2003; received in revised form 28 October 2003; accepted 4 November 2003

Abstract

Reactive oxygen species-mediated cellular injury is involved in the pathogenesis of many diseases, including those affecting the cardiovascular system, such as myocardial ischemia–reperfusion injury, inflammation, and atheroscleosis. Raxofelast (IRFI-016; (±)-5-acetoxy-2, 3-dihydro-4, 6, 7-trimethyl-2-benzofuran-acetic acid) was designed with the aim of maximizing the antioxidant potency of phenols chemically related to vitamin E. The antioxidant activity of raxofelast has been convincingly demonstrated in several in vitro studies and in various models of ischemia–reperfusion injury. In this study, the antiproliferative effects of raxofelast were investigated to determine whether transduction signals and protooncogenes are affected in H_2O_2-stimulated rat aortic smooth muscle cells. In a tetrazolium-based colorimetric assay, the proliferation of rat aortic smooth muscle cells was increased by 3-fold in 0.1% fetal bovine serum/Dulbecco's modified Eagle's medium (DMEM) containing 500 μM H_2O_2, indicating that exogenous 500 μM H_2O_2 was a growth stimulator of rat aortic smooth muscle cells. Exogenous H_2O_2 significantly activated extracellular signal-regulated kinases (ERKs) activity within 30 min and raxofelast inhibited the ERKs activation dose dependently in 500 μM H_2O_2-stimulated rat aortic smooth muscle cells (IC_{50}: 200 μM). Raxofelast reduced the intracellular reactive oxygen species generated by exogenous H_2O_2 in a dose-dependent manner. In 500 μM H_2O_2-stimulated rat aortic smooth muscle cells, raxofelast dramatically attenuated the activation of mitogen-activating protein kinase (MAPK)/ERK kinase 1, 2 (MEK1,2) and protein kinase C (PKC) without affecting Ras expression. Induction of c-*myc* mRNA was significantly reduced dose dependently up to 100 μM by raxofelast in concentrations. These data indicate that the antiproliferative effects of raxofelast in H_2O_2-stimulated rat aortic smooth muscle cells may involve the suppression of intracellular reactive oxygen species formation and the inhibition of ERKs by inactivation through PKC and MEK1,2 and down-regulation of c-*myc* expression, regardless of Ras activation.

Keywords: Raxofelast; Anti-proliferation; Reactive oxygen species; Smooth muscle cell

1. Introduction

Reactive oxygen species such as H_2O_2 have multiple intracellular signaling cascades, depending on their concentrations and cell types. At toxic levels, reactive oxygen species cause DNA strand breaks, lipid peroxidation, and oxidative damage of proteins involved in different pathological processes including neurodegenerative and inflammatory diseases, cancer, and atherosclerosis (Ciccone, 1998; Chapple, 1997; Wiseman and Halliwell, 1997; Dreher and Junod, 1998; Alexander, 1998). Recent evidence has shown that H_2O_2, at non-toxic levels, stimulates cell growth/proliferation and DNA synthesis as a second messenger molecule in mitogen-induced cellular events

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doi:10.1016/j.ejphar.2003.11.012

(Murrell et al., 1990; Rao and Berk, 1992; Zhang et al., 1998). Vascular smooth muscle cell proliferation is one of the major mechanisms of intimal thickening in atherosclerosis, restenosis, and venous bypass graft disease (Luscher et al., 1994; Ross, 1986).

Although clinical trials with vitamin E supplementation to date have been controversial, vitamin E has numerous effects on vascular disease. In vitro, vitamin E has been shown to inhibit platelet adhesion and aggregation and smooth muscle cell proliferation, show anti-inflammatory effects on myocytes, and improve endothelial function (Mabile et al., 1999; Aratri et al., 1999; Ross, 1999; Kaul et al., 2001). With regard to inhibition of smooth muscle cell proliferation, vitamin E inhibited [^{3}H] thymidine incorporation in response to serum, platelet-derived growth factor BB (PDGF-BB), and endothelin in A7r5 smooth muscle cells (Boscoboinik et al., 1991a,b). The inhibition of cell proliferation in A7r5 cells was related to the attenuation of PKC activity (Ozer et al., 1993). Direct inhibition of protein kinase C (PKC) by vitamin E appears likely as several studies showed cellular proliferation was stimulated by either low-density lipoprotein (LDL) or malondialdehyde-modified LDL (Keaney et al., 1999). It is clear that vitamin E has multiple effects promoting vascular homeostasis. Although the data on vitamin E inhibits smooth muscle cell proliferation, it does not provide protection against ischemia–reperfusion injury (Klein et al., 1993). The marvelous lipophilicity of vitamin E has been suggested to slow its incorporation into tissues. Consequently, administration of this agent would result in very low levels of the antioxidant in ischemic–reperfused myocardial tissue (Campo et al., 1998).

Raxofelast (IRFI-016; (±)-5-acetoxy-2,3-dihydro-4,6,7-trimethyl-2-benzofuran-acetic acid) was designed with the aim of maximizing the antioxidant potency of phenols chemically related to vitamin E. The antioxidant activity of raxofelast has been convincingly demonstrated in several in vitro studies and in various models: stimulation of wound healing in genetically diabetic mice (Galeano et al., 2001), inhibition of lipid peroxidation (Altavilla et al., 2001), protection of LDL oxidation (Iuliano et al., 1999), reduction of myocardial infarction (Campo et al., 1998). The molecular mechanisms of the anti-proliferative action of raxofelast remained to be elucidated.

In the present study, we investigated the molecular mechanisms of the anti-proliferative effects of raxofelast to determine how intracellular signaling pathways are regulated in H_2O_2-stimulated rat aortic smooth muscle cells. Our results suggest that the anti-proliferative effects of raxofelast in H_2O_2-stimulated rat aortic smooth muscle cells involve the suppression of intracellular reactive oxygen species formation, the inhibition of extracellular signal-regulated kinases (ERKs), PKC, and mitogen-activating protein kinase (MAPK)/ERK kinase 1,2 (MEK1,2), and the down-regulation of c-*myc* expression.

2. Materials and methods

2.1. Materials

Raxofelast was a gift from Biomedica Foscama (Ferentino, Italy). H_2O_2, collagenase type I and elastase for cell preparations were obtained Sigma (St. Louis, MO, USA). Phospho-ERK, Phospho-PKC, and Ras polyclonal antibodies were from New England Biolabs (Beverly, MA, USA). Dulbecco's modified Eagle's medium (DMEM) and Dulbecco's fetal bovine serums were obtained from Gibco Life Technologies (Gaithersburg, MD, USA). Polyvinylidene difluoride (PVDF) transfer membrane and enhanced chemiluminescence (ECL) Western blotting detection system were purchased from NEN Life Science Products (Boston, MA, USA) and Amersham (Little Chalfont, England), respectively. Reverse Transcription System was obtained from Promega (Madison, WI, USA) and Transwell cell culture chambers using a collagen-treated polycarbonate membrane were purchased from Costar (Cambridge, MA, USA).

2.2. Isolation of rat aortic smooth muscle cells

Rat aortic smooth muscle cells were isolated as previously described (Hwang et al., 2002). Briefly, the aorta was enzymatically isolated from the thoracic aortas from 6 to 8 week-old Sprague–Dawley rats. The aorta was transferred to a plastic tube containing 5 ml of the enzyme dissociation mixture and incubated for 2 h at 37 °C. The suspension was centrifuged (1500 rpm for 10 min) and the pellet was resuspended in DMEM with 10% fetal bovine serum. Cells were cultured over several passages (up to 10). Rat aortic smooth muscle cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 g/ml streptomysin in 75-cm^2 flasks at 37 °C in a humidified atmosphere of 90% air and 10% CO_2 (Forma Scientific, USA).

2.3. Measurement of cell proliferation

Cell proliferation was determined with the Trypan blue dye exclusion method using a hemacytometer. Rat aortic smooth muscle cells were seeded in 24-well culture plates (2.5×10^4 cells/well; well diameter 12 mm) and cultured for 30 min at 37 °C. After cell confluence of ~ 70% was reached, the medium was replaced by 0.1% fetal bovine serum containing DMEM and H_2O_2. After a 72-h incubation, cells were harvested from dishes using a 0.1% w/v trypsin solution, and viability was examined by the Trypan blue dye exclusion test. The number of viable cells was counted using a hemacytometer. For estimation of the proliferation rate, cells were quantified by the nonradioactive colorimetric assay WST-1 (Boehringer Mannheim), based on the cleavage of a tetrazolium salt, as recommended by the manufacturer.

2.4. Measurement of intracellular reactive oxygen species generation

Rat aortic smooth muscle cells were labeled with 2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probe, CA, USA) (Watson et al., 2001). The probe H_2DCFDA (5 μM) enters the cell and the acetate group on H_2DCFDA is cleaved by cellular esterases, trapping the nonfluorescent 2′, 7′-dichlorofluorescin (DCFH) inside. Subsequent oxidation by reactive oxygen species yields the fluorescent product DCF. The dye, when exposed to an excitation wavelength of 480 nm, emits light at 535 nm only when it has been oxidized. Labeled rat aortic smooth muscle cells were examined using a luminescence spectrophotometer for oxidized dye. The quiescent cells were treated with raxofelast for 3 h before labeling with H_2DCFDA.

2.5. Immunoblot analysis

Confluent rat aortic smooth muscle cells were cultured for 48 h in serum-free DMEM and were pre-treated with raxofelast (0–200 μM) for 3 h at 37 °C. The cells were then treated with the stimulator H_2O_2 (500 μM) for 30 min at 37 °C. At the end of the various treatments, cells were lysed in lysis buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 1 mM Na_2VO_4, 1 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate, 1 μg/ml leupeptin) for 15 min on ice. Soluble extracts were prepared by centrifugation at 16,000 × *g* for 15 min at 4 °C. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gel. After electrophoresis, proteins were electrotransferred to methanol-treated PVDF membranes. The blotted membranes were washed twice with water and blocked by incubation with 3% nonfat dry milk in PBS buffer (8.0 g NaCl, 0.2 g KCl, 1.5 g NaH_2PO_4, 0.2 g K_2HPO_4 per liter) for 20 min at room temperature. For Western blot analysis of the expression and phosphorylation of ERK1,2 (42 and 44 kDa), PKC (82 kDa), and MEK1 (43 kDa), the membranes were incubated with anti-ERK antibody, anti-MEK1,2 antibody, and PKC antibody followed by peroxidase goat anti-rabbit immunoglobulin G and detected by ECL. For the Western blot analysis of Ras, antibody against Ras (21 kDa) was used.

2.6. RT-PCR analysis

The expression levels of c-*fos*, c-*jun*, and c-*myc* mRNA were analyzed by the reverse transcription polymerase chain reaction (RT-PCR) technique, as previously described (Hwang et al., 2002). For the RNA preparation, confluent rat aortic smooth muscle cells were cultured for 48 h in serum-free DMEM and were pretreated with raxofelast (0–200 μM) for 3 h at 37 °C. The cells were then treated with the stimulator H_2O_2 (500 μM) for 30 min at 37 °C. Total RNA was prepared by Ultraspect™-II RNA system (Biotecx Laboratories, USA) and single-stranded cDNA was then synthesized from isolated total RNA by Avian myeloblastosis virus (AMV) reverse transcriptase. A 20-μl reverse transcription reaction mixture containing 1 μg of total RNA, 1 × reverse transcription buffer (10 mM Tris–HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100), 1 mM deoxynucleoside triphosphates (dNTPs) 0.5 unit of RNase inhibitor, 0.5 μg of oligo$(dT)_{15}$, and 15 units of AMV reverse transcriptase were incubated at 42 °C for 15 min, heated at 99 °C for 5 min, and then incubated at 0–5 °C for 5 min. PCRs were performed for 35 cycles with 3′ and 5′ primers based on the sequences of c-*myc* gene primers (5′-gaagtgaccgactgttctatgact-3′ and 5′-cgcaaccagtcaagttctcaagtt-3′). The actin pri-

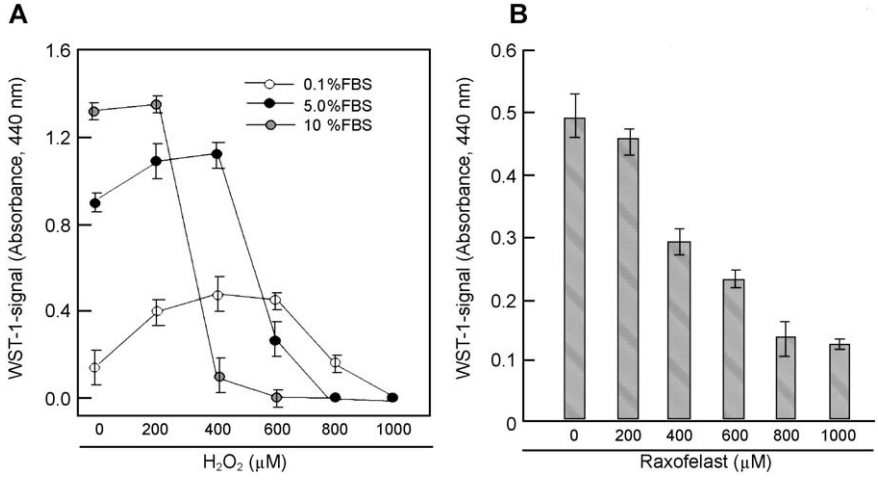

Fig. 1. Effects of H_2O_2 and raxofelast on the proliferation of rat aortic smooth muscle cells. (A) Quiescent cells (2.5×10^4 cells per well) were stimulated with various concentrations of serum and H_2O_2 for 72 h. (B) Quiescent cells were pretreated with various concentrations of raxofelast for 3 h and were further incubated with 500 μM H_2O_2 for 72 h. Cell viability was quantified by the nonradioactive colorimetric assay WST-1. Data denote the means ± S.E. of 3–4 replicate measurements in three different cell cultures.

mers (5′-catcacatactcacaacgctcaac-3′ and 5′-catagcacgatggt cgattgtcgt-3′) were used as the internal standard. The signal intensity of the amplification product was normalized to its respective actin signal intensity.

2.7. Statistical analysis

The data are expressed as means ± S.E. P values < 0.05 were considered to be statistically significant (analysis of variance and Student's t-test).

3. Results

3.1. Effect of raxofelast on exogenous H_2O_2-stimulated rat aortic smooth muscle cell proliferation

To investigate the optimal conditions for the stimulation by exogenous H_2O_2 of the proliferation of rat aortic smooth muscle cells, the cells were cultured with various concentrations of fetal bovine serum and H_2O_2. At a high concentration of fetal bovine serum, H_2O_2 was not an effective stimulator of cell proliferations. H_2O_2 stimulated the proliferation of cells in a dose-dependent manner up to 500 μM in 0.1% fetal bovine serum/DMEM (Fig. 1A), by an average of 3-fold over control levels. At concentration higher than 500 μM H_2O_2, exogenous H_2O_2 played a role in cell death. On the basis of the concentration–response data, cells were exposed to exogenous H_2O_2 at 500 μM in most of the later experiments. To examine the effect of raxofelast on the proliferation of H_2O_2-stimulated rat aortic smooth muscle cells, the cells were incubated with various concentrations of raxofelast for 3 h. Raxofelast inhibited H_2O_2-stimulated proliferation in dose-dependent manner with an IC_{50} value of about 500 μM (Fig. 1B).

3.2. Effect of raxofelast on ERK activation in exogenous H_2O_2-stimulated rat aortic smooth muscle cells

Among MAP kinases, ERKs are activated by growth factors and are involved in both cell proliferation and differentiation. We first examined whether exogenous H_2O_2 activates ERKs in rat aortic smooth muscle cells. Growth-arrested cells were treated with 500 μM H_2O_2 for various times. Fig. 2A shows that H_2O_2 caused a time-dependent increase in ERK activation, with maximal levels at 30 min. Compared with untreated cells, cells stimulated with 500 μM H_2O_2 for 30 min showed a 7-fold increase in ERK activation. To investigate the inhibitory effect of raxofelast on ERK activation in H_2O_2-stimulated cells, raxofelast was added at various concentrations for 3 h. Raxofelast treatment reduced the phosphorylated ERKs levels induced by H_2O_2 to the basal level in dose-dependent

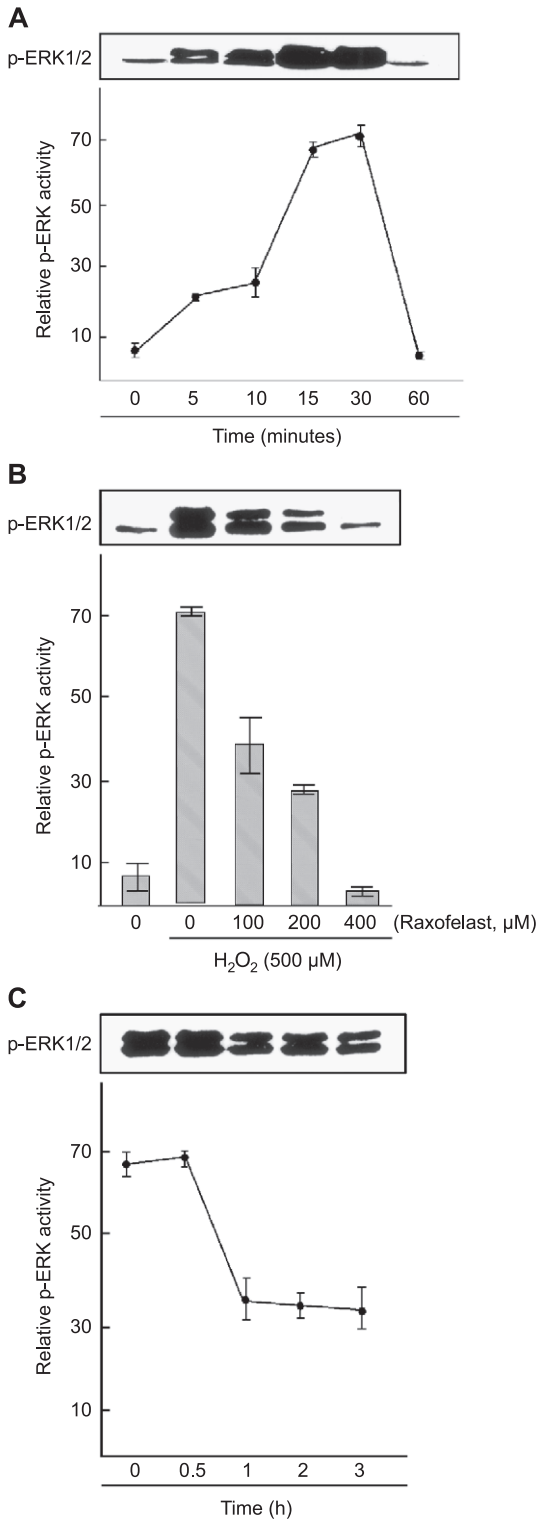

Fig. 2. Effects of H_2O_2 and raxofelast on the phosphorylation of ERKs in rat aortic smooth muscle cells. Quiescent cells were incubated with 500 μM H_2O_2 for the indicated times (A). After cells were pretreated with various concentrations of raxofelast for 3 h, the cells were further stimulated with 500 μM H_2O_2 for 30 min (B). After cells were pretreated with 400 μM raxofelast for the indicated times, the cells were further stimulated with 500 μM H_2O_2 for 30 min (C). The phosphorylation of ERKs was measured by immunoblot analysis with anti-phospho-ERK antibody and each signal was quantified by scanning densitometry. Data denote the means ± S.E. of 2–3 replicate measurements in three different cell cultures.

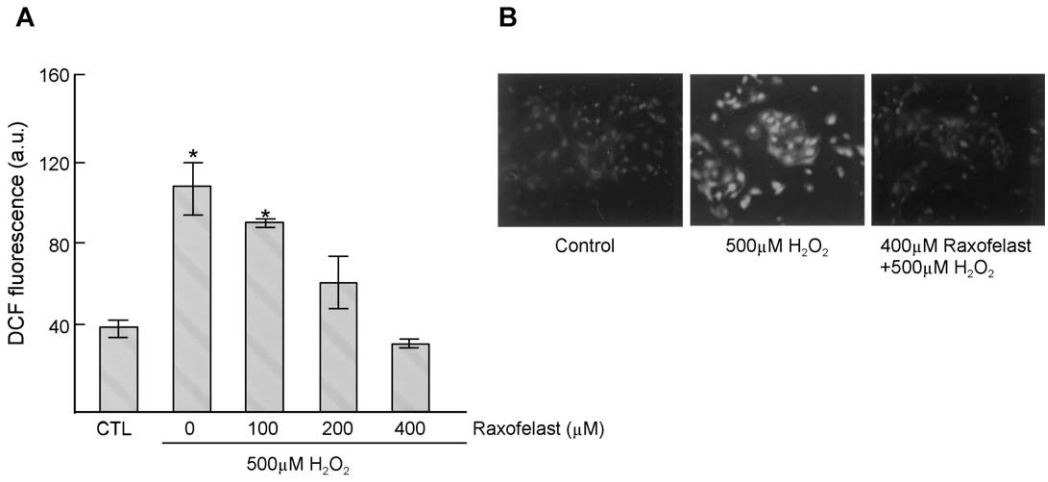

Fig. 3. Effect of raxofelast on the formation of intracellular reactive oxygen species in rat aortic smooth muscle cells. After quiescent cells were pretreated with 400 μM raxofelast for 3 h, cells were incubated with 500 μM H_2O_2 for 15 min. Cells were further treated with H_2DCFDA (5 μM) for 10 min and the oxidized dye, DCF, was measured using a luminescence spectrophotometer. Data denote the means ± S.E. of 2–3 replicate measurements in three different cell cultures. *$P < 0.05$, when compared with the control value.

manner (Fig. 2B). To further investigate the influence of pretreatment with raxofelast, cells were pretreated with 200-μM raxofelast for different times. In Fig. 2C, ERK activity was reduced by pretreatment with raxofelast up to 1 h, but no difference in ERK activity was seen after a 1-h treatment. This suggests that raxofelast inhibits initial signals related to cell growth, leading to suppression of protein synthesis and protooncogene transcription.

3.3. Effect of raxofelast on the formation of intracellular reactive oxygen species in rat aortic smooth muscle cells

Exogenous H_2O_2 can pass through the cell membrane freely and stimulate MAP kinases to cause the proliferation of different cells. To investigate the effect of raxofelast on the formation of intracellular reactive oxygen species, cells were pretreated with various concentrations of raxofelast. Fig. 3A shows that raxofelast reduced the formation of reactive oxygen species generated from exogenous H_2O_2 in a dose-dependent manner. Fig. 3B shows the suppressive effect of raxofelast on intracellular reactive oxygen species generated by exogenous H_2O_2-stimulated rat aortic smooth muscle cells. The inhibitory effect of raxofelast on the ERK activity was directly related to the suppression of intracellular reactive oxygen species generation in H_2O_2-stimulated rat aortic smooth muscle cells.

3.4. Effect of raxofelast on upstream regulators in the suppression of ERK activity

The ERKs are activated by various upstream regulators, Ras, PKC, and/or MEK1,2. To determine which of these kinases is responsible for the inhibition of ERKs, the phosphorylation level of these upstream regulators was investigated. As shown in Fig. 4A, the activity of PKC and MEK1,2 was decreased by raxofelast in H_2O_2-stimulated rat aortic smooth muscle cells, whereas the expression of Ras was not affected. This indicates that the antiproliferative intracellular signaling mechanism of raxofelast involves the PKC/MEK1,2/ERK cascade.

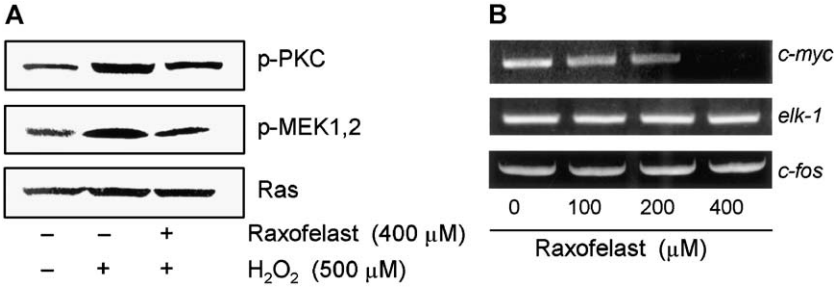

Fig. 4. Effects of raxofelast on the upstream regulators of ERKs phosphorylation and early gene expression in rat aortic smooth muscle cells. (A) After quiescent cells were pretreated with 400 μM raxofelast for 3 h, cells were incubated with 500 μM H_2O_2 for 15 min. Equivalent amounts of protein lysates from cells were subjected to SDS-PAGE, transferred to a PVDF membrane, and incubated with antibody. The immunoreactive protein was visualized by use of an ECL detection system. (B) The expression of early genes was analyzed by RT-PCR amplification. Amplification products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

3.5. Effects of raxofelast on early gene expression

To further confirm the involvement of the PKC/MEK1,2/ERKs cascade in the antiproliferative response induced by raxofelast, the mRNA levels of three protooncogenes, c-*fos*, c-*myc* and *elk-1*, in H_2O_2-stimulated rat aortic smooth muscle cells treated with raxofelast were measured using the RT-PCR technique. In Fig. 4B, the expression of all three mRNAs at different concentration of raxofelast is shown. The mRNA levels of c-*fos* and *elk-1* were not significantly affected by raxofelast. However, the level of c-*myc* mRNA was significantly reduced in a dose-dependent manner, indicating that the PKC/MEK1,2/ERK cascade is coupled to the transcription of the c-*myc* gene in rat aortic smooth muscle cells.

4. Discussion

Reactive oxygen species have indiscriminate effects on a variety of cellular targets, including oligonucleotides, lipids, and proteins, resulting in cell death. At lower concentrations, however, reactive oxygen species activate very specific pathways that in turn regulate the expression of genes influencing cell survival (Adderley and Fitzgerald, 1999). Exogenous reactive oxygen species play a key role in mediating smooth muscle growth and proliferation, which are important in the pathogenesis of intimal thickening in atherosclerosis, restenosis, and venous bypass graft disease. Activation of ERKs is a key step in the cascade mediating cell proliferation in response to a variety of extracellular signals, including epidermal growth factors, platelet-derived growth factor, and phorbol esters (Zhang et al., 1998). The present study provides evidence that exogenous H_2O_2 (500 μM) activates ERKs and early gene c-*myc* in rat aortic smooth muscle cells. These findings are consistent with the data of Zhang et al. (1998) and Guyton et al. (1996) in pulmonary artery smooth muscle cells. It has been suggested that the activation of ERKs by exogenous H_2O_2 is mediated through both PKC-dependent and -independent mechanisms. Several observations in our study suggest that exogenous H_2O_2 (500 μM) activates PKC and MEK1,2, leading to activation of ERKs. These results are partly in accordance with data showing that H_2O_2-induced activation of ERKs is PKC-dependent and PKC-independent (Zhang et al., 1998; Whisler et al., 1995; Abe et al., 1994)

Vitamin E has been reported to inhibit smooth muscle cell proliferation in culture (Kaul et al., 2001). This antiproliferative effect on smooth muscle involves PKC phosphorylation. Evidence for vitamin E inhibition is based almost entirely on in vitro data, and experience in vivo has thus far been mixed. Acute administration of vitamin E, however, is not feasible due to its high lipophilicity and resulting slow tissue incorporation (Campo et al., 1998; Klein et al., 1993). Thus, vitamin E analogues, with a less lipophilic character, have been developed.

In the present study, we demonstrated that raxofelast inhibits the proliferation and the formation of intracellular reactive oxygen species in H_2O_2-stimulated rat aortic smooth muscle cells. The antiproliferative mechanism of raxofelast is associated with the inhibition of ERKs activated by H_2O_2 stimulation; raxofelast affects up-stream regulators of ERK, PKC, and MEK1,2 activity and early gene, c-*myc*, transcription. It could be suggested that the antiproliferative mechanisms of raxofelast may be similar to those of vitamin E in PKC-dependent inhibition of proliferation.

Raxofelast was selected with the aim of maximizing the antioxidant potency of phenols chemically related to vitamin E, having a more hydrophilic nature than vitamin E. Raxofelast has potent protective effects against oxidative damage in in vitro studies (Mattoli et al., 1991) and in various models of oxyradical-mediated ischemia–reperfusion injury (Campo et al., 1994). It was also reported that raxofelast may be associated with a decrease in peroxynitrite formation, which may lead to prevention of the activation of poly (ADP-ribose) synthetase in inflammation (Cuzzocrea et al., 1999).

In conclusion, our results demonstrate that the antiproliferative effects of raxofelast in H_2O_2-stimulated rat aortic smooth muscle cells were mediated by PKC/MEK1,2/ERK1,2, independent of Ras, and could be attributed to the down-regulation of c-*myc* expression. This may provide a potential therapeutic approach to prevent myocardial ischemia–reperfusion injury, inflammation, and atheroscleosis.

Acknowledgements

This study was supported by a grant of the Korea Health 21 R and D Project, Ministry of Health and Welfare, Republic of Korea. (HMP-00-GN-01-0001).

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