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Inhibitory effect of pentalenolactone on vascular smooth muscle cell proliferation

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Abstract

The effect of pentalenolactone, an inhibitor of glyceraldehyde-3-phosphate dehydrogenase, on rat vascular smooth muscle cell proliferation was studied. Addition of pentalenolactone together with serum to quiescent cells dose-dependently inhibited cell proliferation and DNA synthesis. This inhibition was not associated with cell death. When quiescent cells were stimulated with serum and then treated with pentalenolactone, the inhibitory effect on the DNA synthesis declined gradually. A similar result was obtained when PD 98059 (2′-amino-3′-methoxyflavone), an inhibitor of extracellular signal-regulated kinase1/2 (ERK1/2) kinase (MEK1/2), was added to the cells after serum stimulation. Pentalenolactone inhibited serum or protein kinase C activator (phorbol 12,13-dibutyrate)-induced phosphorylation of ERK1/2 and MEK1/2. In contrast, pentalenolactone had little effect on platelet-derived growth factor receptor autophosphorylation. Taken together, these results indicate that pentalenolactone inhibits vascular smooth muscle cell proliferation, and that this inhibition appears to be mediated by inhibition of the ERK1/2 cascade.

Keywords: Pentalenolactone; Smooth muscle cell, vascular; ERK1/2; MEK1/2; Cell proliferation

1. Introduction

Pentalenolactone, a sesquiterpenoid, was first isolated from *Streptomyces* sp. in 1957 and has been shown to be active against a wide range of microorganisms including Gram-positive and Gram-negative bacteria, fungi and protozoa (Koe et al., 1957; English et al., 1957). This substance inhibits glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) from a wide variety of sources including *Escherichia coli*, *Bacillus subtilis*, *Trypanosoma brucei*, yeast and various mammalian cells. The antibiotic activity of pentalenolactone is thought to be due to inhibition of glycolysis (Hartmann et al., 1978; Mann and Mecke, 1979; Duszenko et al., 1982; Lambeir et al., 1991; Cane and Sohng, 1989). Pentalenolactone has also been shown to have antitumour activity against Sarcoma 180 in mice (Takahashi et al., 1983). Up to now, however, the mechanisms underlying the antitumour activity of pentalenolactone in mammalian cells have yet to be elucidated.

The mitogen-activated protein kinase (MAPK) family, which includes the extracellular signal-regulated kinases (ERKs), c-Jun NH_2-terminal kinases (JNKs, also called SAPKs), and p38 MAPK, is an important player in the early intracellular signal transduction and is activated by a variety of stimuli, such as growth factors and cellular stresses (Davis, 1993; Kyriakis et al., 1994; Han et al., 1994; Derijard et al., 1994). Among the MAPK family, ERK1/2 has been implicated in vascular smooth muscle cell proliferation (Duff et al., 1992; Watson et al., 1993; Xi et al., 1999; Pukac et al., 1992).

In the present study, we show that pentalenolactone inhibits vascular smooth muscle cell proliferation. We also provide evidence that this inhibition may be associated with inhibition of the ERK1/2 cascade.

2. Methods

2.1. Cells

Rat vascular smooth muscle cells were isolated using a modification of the technique described by Chamley et al. (1977). The rat thoracic aorta was dissected from

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Sprague–Dawley rats (250–300 g; Japan SLC, Shizuoka, Japan) and incubated in Dulbecco's modified Eagle's medium (DMEM; Nissui, Pharmaceutical, Tokyo, Japan) containing 1 mg/ml collagenase (Type I; Worthington Biochemical, Lakewood, NJ) for 20 min at 37°C. After removal of the adventitia, small fragments of outer membrane and endothelial cells, the aorta was minced and incubated in DMEM containing collagenase and 1 mg/ml elastase (Type III; Sigma, St. Louis, MO) for 1.5 h at 37°C. The freshly isolated cells were resuspended in DMEM containing 4 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 8 μg/ml tylosin and 10% fetal bovine serum (serum; GIBCO-BRL, Grand Island, NY) and grown in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Most experiments were performed with rat vascular smooth muscle cells after serum starvation. Cells were grown to 80% confluence in DMEM supplemented with 10% serum and transferred to DMEM containing 0.1% bovine serum albumin (fraction V; Sigma) or 0.5% serum for 72 h. Expression of α-smooth muscle actin was determined by Western blotting and was confirmed in all vascular smooth muscle cells used.

Swiss 3T3 cells were purchased from Dainippon Pharmaceutical (Tokyo, Japan). Swiss 3T3 cells were grown in the same medium used for vascular smooth muscle cell.

2.2. Measurement of cell proliferation and DNA synthesis

Rat vascular smooth muscle cell proliferation was measured by determining cell number. Cells were seeded at a concentration of 4×10^3 cells/well in 12-well plates and grown in DMEM containing 5% serum in the presence of vehicle (1% ethanol) or pentalenolactone. At 2, 4 and 6 days after seeding, the cells were suspended by digestion with 0.1% trypsin–1 mM EDTA, and the cell number was counted by a hemocytometer.

DNA synthesis in vascular smooth muscle cells was evaluated using the 5-bromo-2′-deoxy-uridine (BrdU) incorporation assay kit (Boehringer Mannheim Biochemica, Mannheim, Germany). Cells were seeded in 24-well plates. When the dose-dependent effect of pentalenolactone on DNA synthesis was examined, quiescent cells were incubated with serum in the presence or absence of various concentrations of pentalenolactone for 20 h, and then labeled with 10 μM BrdU for 4 h. When the cell cycle-dependent effect of pentalenolactone, PD98059 (2′-amino-3′-methoxyflavone), or olomoucine was investigated, each agent was added at the indicated time point after the addition of serum, and the cells were labeled with BrdU during the last 4 h of incubation (20–24 h after stimulation with serum). After labeling with BrdU, the cells were fixed with 70% ethanol containing 0.5 M HCl for 30 min at −20°C. Following fixation, the cells were incubated with buffer containing nucleases for 30 min at 37°C, and peroxidase-labeled antibody to BrdU was then added to each well. After addition of the peroxidase substrate, the absorbance was determined at 405 nm, with a reference wavelength of 490 nm, using a microtiter plate reader (model 550; Nippon Bio-Rad Lab., Tokyo, Japan).

Vascular smooth muscle cell viability was determined by the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate (WST-1) assay (Ishiyama et al., 1995). Quiescent cells were incubated with serum in the presence or absence of various concentrations of pentalenolactone for 24 h and a mixture of WST-1 and 1-methoxy-5-methylphenazinium methosulphate was then added to each well (final concentrations: WST-1, 0.5 mM; 1-methoxy-5-methylphenazinium methosulphate, 0.02 mM). After incubation for 1 h, the absorbance was measured at 405 nM, with a reference wavelength of 655 nm.

The cell numbers and viability of Swiss 3T3 cells were determined by 3-(4,5-dimethyl-thiazoyl-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Green et al., 1984). Briefly, cells cultured in 96-multi well plates received 50 μl of 1 mg/ml of MTT solution and were incubated at 37°C for 4 h. The medium was discarded, the cells were dissolved in DMSO, and the absorbance at 540 nm was measured.

2.3. Determination of time course of cell cycle progression in vascular smooth muscle cells

To determine the time course of BrdU incorporation, quiescent vascular smooth muscle cells were pulse-labeled with BrdU every 4 h after serum stimulation.

To define the restriction point, quiescent vascular smooth muscle cells were incubated in the presence of 5% serum for various time periods, and then were washed three times with DMEM containing 0.1% bovine serum albumin and further incubated in the medium without serum up to 24 h. BrdU uptake was measured for 4 h in the end of incubation.

2.4. Evaluation of phosphorylations of the platelet-derived growth factor (PDGF) receptor, ERK and MEK

Quiescent vascular smooth muscle cells were stimulated with serum, phorbol 12,13-dibutyrate (PDB), or PDGF-BB, in the absence or presence of pentalenolactone, as indicated. Following stimulation, cells in 100-mm dishes were rinsed quickly with ice-cold Tris-buffered saline (TBS; 25 mM Tris, pH 7.4, 150 mM NaCl) containing 1 mM Na_3VO_4 and then lysed in lysis buffer (50 mM Tris, pH 7.4, 50 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM Na_3VO_4, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml antipain, 100 μg/ml benzamidine, 10 μg/ml aprotinin, 100 μg/ml soybean trypsin inhibitor, 1% glycerol) for 20 min at 4°C. The cells were then scraped off the dish, centrifuged at $12{,}000 \times g$ for 10 min, and the protein concentration of the supernatants was determined by the DC protein assay (Nippon Bio-Rad). The remaining supernatants were combined with 4× SDS-polyacrylamide gel electrophoresis

(SDS-PAGE) sample buffer (0.5 M Tris, pH 6.8, 50% glycerol, 8% SDS, 0.4 M dithiothreitol, 0.05% bromophenolblue) and heated at 95°C for 3 min. The samples were then frozen at −80°C until use. After separation by SDS-PAGE, the proteins were transferred to nitrocellulose membrane and analyzed. The membrane was incubated in blocking solution and then with primary antibodies for 1.5 h at 37°C. Excess primary antibody was removed by washing the membranes in TBS containing 0.05% Tween 20. The blot was incubated with appropriate secondary antibodies for 1 h at 37°C, and the membrane was then washed and proteins were detected by the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Tokyo Japan). For repeated immunoblotting, the membranes were stripped in 50 mM Tris, pH 6.5, 100 mM 2-mercaptoethanol and 2% SDS for 30 min at 50°C.

2.5. Determination of glycolysis

Cellular glycolysis was calculated from lactate production (Duszenko et al., 1982). Swiss 3T3 cells were incubated in the culture medium from which phenol red was deprived for 4 h in the absence or presence of various concentrations of pentalenolactone. Lactate content in conditioned medium was measured using commercially available kit (Sigma).

2.6. Chemicals and reagents

Pentalenolactone was isolated from culture broth of *Streptomyces nashvillensis* according to the method reported by Hartmann et al. (1978). The rabbit polyclonal anti-phospho-ERK1/2 antibody specific for dual-phosphorylated ^{202}Thr and ^{204}Tyr of ERK1/2, the rabbit polyclonal anti-phospho-MEK1/2 antibody specific for dual-phosphorylated $^{217/221}$Ser of MEK1/2, and the rabbit polyclonal anti-MEK1/2 antibody which detects phosphorylation-state independent MEK1/2 were purchased from New England Biolabs (Beverly, MA). The monoclonal anti-phosphotyrosine antibody was from Upstate Biotechnology (clone 4G10; Lake Placid, NY). Horseradish peroxidase-conjugated sheep anti-mouse and donkey anti-rabbit secondary antibodies were from Amersham Pharmacia Biotech. PD98059 and olomoucine were from Calbiochem® (La Jolla, CA). Human recombinant PDGF-BB was from Boehringer Mannheim. All other chemicals and reagents were purchased from Sigma or Wako (Osaka, Japan).

3. Results

3.1. Pentalenolactone inhibits cell proliferation and DNA synthesis

The number of rat vascular smooth muscle cells in culture was determined after 2, 4 or 6 days stimulation with serum. The results were shown in Fig. 1A. Treatment of cells with 5% serum and vehicle for pentalenolactone (1% ethanol) increased the cell number in a time-dependent fashion. The addition of 0.36 μM pentalenolactone together with serum completely inhibited the increase in cell number. At a dose of 0.11 μM, pentalenolactone also reduced the increase in cell number for 2 days, but longer treatment with pentalenolactone failed to inhibit it. The decreased inhibitory effect of a low concentration of pentalenolactone may be due to the reduced concentration of pentalenolactone in the medium, because it is possible that this compound is partially and slowly adsorbed by serum (Duszenko et al., 1982).

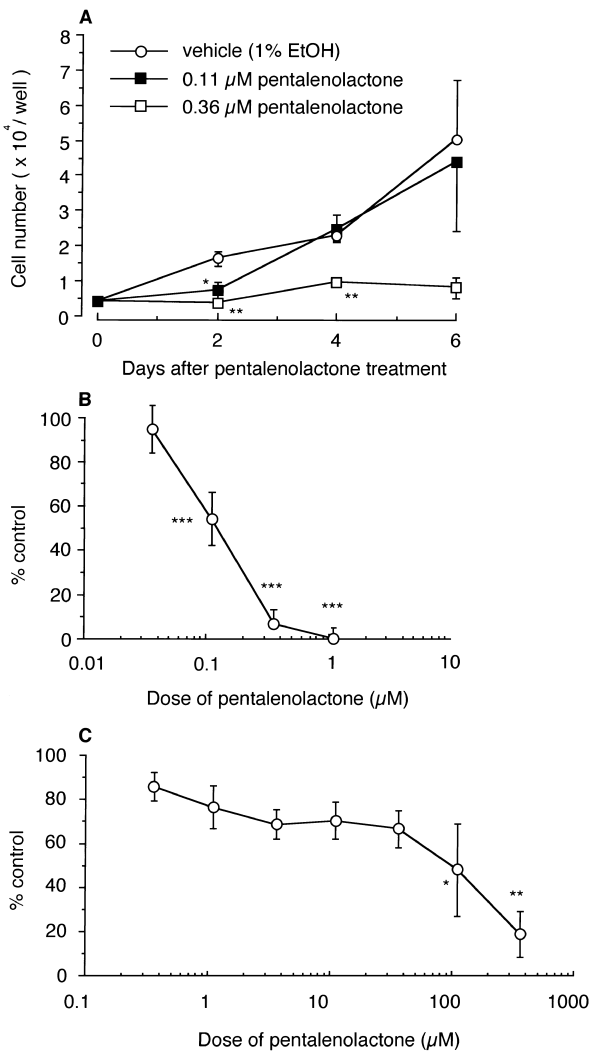

Fig. 1. Effect of pentalenolactone on vascular smooth muscle cell proliferation (A), DNA synthesis (B) and metabolic reduction of WST-1 (C). Each experiment was performed in duplicate (A and C) or in triplicate (B) and repeated two (A and C) or three times (B). Values are presented as means ± S.E. In B and C, data are expressed as percent of the mean control value obtained from vascular smooth muscle cells incubated with medium in the absence of pentalenolactone. *, ** and *** represent the significance level of $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively, tested for the difference between with and without pentalenolactone (Dunnett's method).

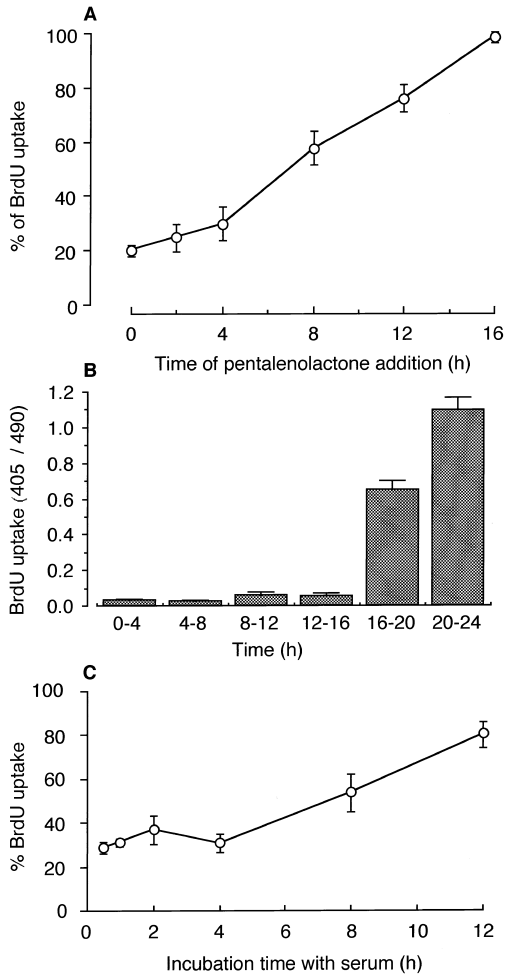

Fig. 2. (A) Effect of treatment with pentalenolactone after serum stimulation on DNA synthesis. Pentalenolactone (0.36 μM) was added at the indicated time point (h) after the addition of serum (zero time point). Data are expressed as percent of the mean value obtained from cells treated with pentalenolactone 16 h after serum stimulation. (B) Time course of BrdU incorporation into DNA in serum-stimulated rat vascular smooth muscle cells. Quiescent vascular smooth muscle cells were stimulated with serum at zero time point and pulse-labeled with BrdU during the indicated periods of time. (C) For defining the restriction point, quiescent vascular smooth muscle cells were stimulated with serum at zero time point and after the indicated various time periods vascular smooth muscle cells were washed and immersed with starvation medium. Data are expressed as percent of the mean value obtained from cells treated with serum for 24 h. In A–C, each experiment was performed in triplicate and repeated twice. Values are presented as means ± S.E.

To examine the effect of pentalenolactone on DNA synthesis, the uptake of BrdU into rat vascular smooth muscle cells was measured. Without serum stimulation, vascular smooth muscle cells did not incorporate BrdU into DNA, but addition of serum (5%) to quiescent vascular smooth muscle cells resulted in the incorporation of BrdU into DNA. When pentalenolactone and serum were administered together, serum-induced BrdU uptake was inhibited. After the relative BrdU uptake of cells at each dose of pentalenolactone had been calculated with reference to the mean BrdU incorporation without pentalenolactone (in the presence of vehicle), the relative BrdU uptake was plotted against a logarithmic scale of dose (Fig. 1B). It can be seen that pentalenolactone acted in a dose-dependent fashion, with a 50% inhibitory concentration of 0.12 μM.

The cytotoxicity of pentalenolactone was then determined by the WST-1 assay. As shown in Fig. 1C, treatment of rat vascular smooth muscle cells with a pentalenolactone concentration of 100 μM or more for 24 h significantly reduced cell viability. The 50% cytotoxic concentration of pentalenolactone was 100 μM. Therefore, the inhibitory effect of pentalenolactone on cell proliferation and DNA synthesis did not appear to be due to cytotoxicity.

3.2. Mode of action of pentalenolactone on DNA synthesis

To investigate the site of action of pentalenolactone within the mitogenic signal transduction induced by serum, pentalenolactone was added at various time points after the addition of 5% serum (zero time point), and BrdU uptake of rat vascular smooth muscle cells was measured for 20–24 h after serum stimulation (Fig. 2A). The inhibitory

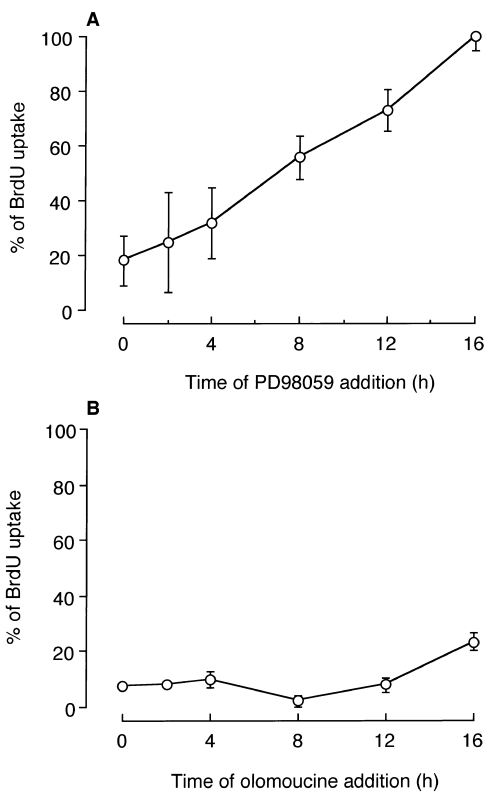

Fig. 3. Effect of treatment with PD98059 (A) or olomoucine (B) after serum stimulation on DNA synthesis. PD98059 (100 μM) or olomoucine (200 μM) was added at the indicated time point (h) after the addition of serum (zero time point). Data are expressed as percent of the mean value obtained from cells treated with PD98059 or olomoucine 16 h after serum stimulation. Values are presented as means ± S.E. Each experiment was performed in triplicate and repeated twice.

effect of pentalenolactone decreased as the time between treatment of cells with pentalenolactone and stimulation with serum increased.

To determine the kinetics of DNA synthesis, the time course of BrdU uptake of the rat vascular smooth muscle cells was examined after stimulation with serum (Fig. 2B). As serum stimulation increased BrdU uptake after a lag-period (G1 phase) of 16 h, the G1/S boundary was estimated to be around 16 h.

To clear the restriction point of the rat vascular smooth muscle cells, BrdU uptake was measured in quiescent cells stimulated with serum for various time periods (Fig. 2C). Cells stimulated with serum for up to 4 h did not take up BrdU, but longer treatment with serum resulted in the incorporation of BrdU. This indicates that quiescent rat vascular smooth muscle cells pass the restriction point 8 h after readdition of serum.

3.3. Inhibitory effects of PD98059 and olomoucine on DNA synthesis

To compare the site of action of pentalenolactone with that of MEK or that of Cdk2 inhibitor, PD98059 (Pang et al., 1995) or olomoucine (Vesely et al., 1994) was added at various time points after the addition of 5% serum (zero time point), and BrdU uptake of vascular smooth muscle cells was measured for 20–24 h after serum stimulation. The inhibitory effect of PD98059 decreased as the time between treatment of cells with PD98059 and stimulation with serum increased (Fig. 3A). This result was similar to that obtained with pentalenolactone. In contrast to pentalenolactone and PD98059, olomoucine fully inhibited the 5% serum-induced vascular smooth muscle cell proliferation, even when it was added at 12 h after the treatment with serum (Fig. 3B).

3.4. Effect of pentalenolactone on phosphorylation of ERK1/2

As the mode of action of pentalenolactone on DNA synthesis was similar to that of PD98059, the effect of pentalenolactone on phosphorylation of ERK1/2 was examined. Pretreatment of cells with pentalenolactone for 4 h dose-dependently inhibited serum-induced ERK1/2 phos-

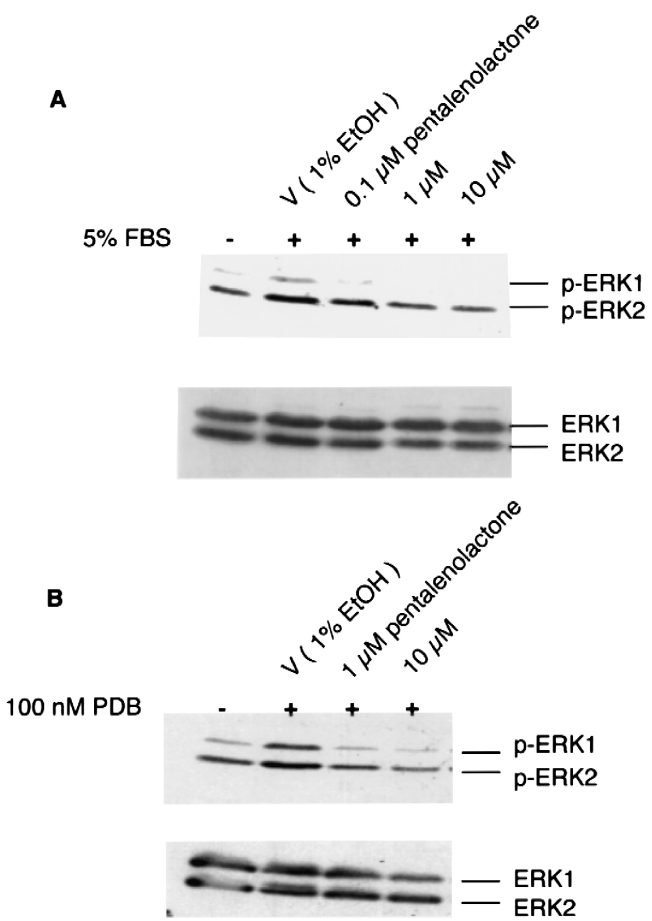

Fig. 4. Effect of pentalenolactone on phosphorylation of ERK1/2 by serum (A) or PDB (B) in vascular smooth muscle cells. Quiescent vascular smooth muscle cells were incubated with or without pentalenolactone at the indicated concentration for 4 h. Cells were then stimulated briefly with serum (1 min at 37°C) or PDB (10 min at 37°C). Extracts were subjected to SDS-PAGE in an 11% acrylamide gel and immunoblotting with anti-phospho-ERK1/2 antibody. The same membrane was then stripped and reprobed with anti-ERK1/2 antibody. p-ERK and ERK indicate the phosphorylated form of ERK and total ERK, respectively. Similar results were obtained in three (A) or six (B) separate experiments.

phorylation (Fig. 4A). In contrast, pentalenolactone had no effect on the amount of ERK1/2 protein.

It has been shown that activation of protein kinase C (PKC) by phorbol ester also phosphoryoates ERK1/2 (Kolch et al., 1991; Grammer and Blenis, 1997). The effect of pentalenolactone on the protein kinase C-activated ERK1/2 phosphorylation was, therefore, examined. When quiescent vascular smooth muscle cells were stimulated with 100 nM PDB, an activator of conventional and novel protein kinase C (Lee and Severson, 1994), marked phosphorylation of ERK1/2 was observed (Fig. 4B). Pretreatment of cells with pentalenolactone for 4 h fully inhibited PDB-induced ERK1/2 phosphorylation (Fig. 4B).

3.5. Effect of pentalenolactone on phosphorylation of MEK1/2

It has been reported that ERK1/2 is phosphorylated and activated by the upstream kinase MEK1/2 (Meloche et al., 1992a,b). Therefore, the effect of pentalenolactone on serum-induced phosphorylation of MEK1/2 was determined. When quiescent vascular smooth muscle cells were stimulated with 5% serum, phosphorylation of MEK1/2 was observed (Fig. 5A). Pretreatment of cells with pentalenolactone for 4 h inhibited serum-induced phosphorylation of MEK1/2, but had no effect on the amount of MEK1/2 protein (Fig. 5A).

PDB also induced phosphorylation of MEK1/2 (Fig. 5B). Pentalenolactone similarly inhibited PDB-induced MEK1/2 phosphorylation, although it had no effect on the amount of MEK1/2 protein (Fig. 5B).

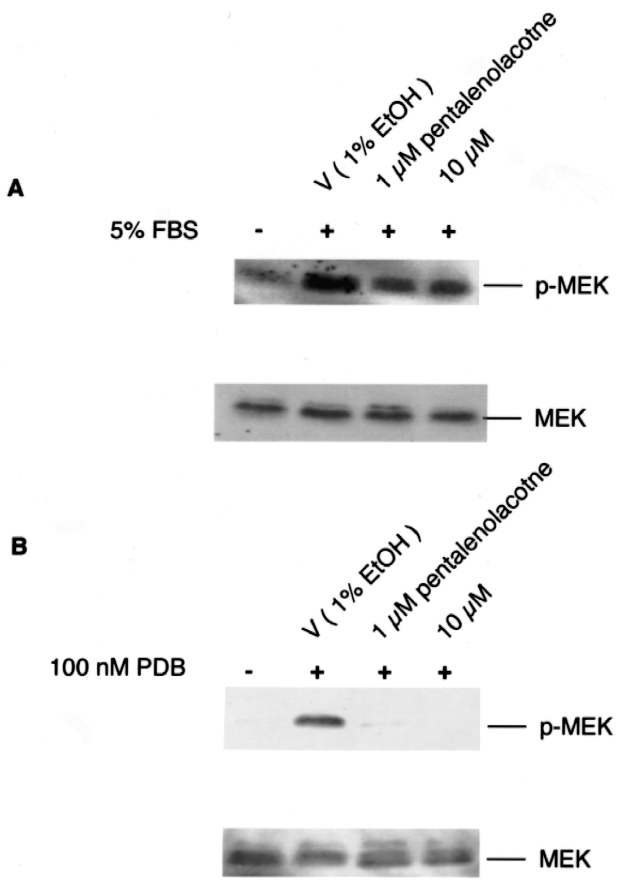

Fig. 5. Effect of pentalenolactone on phosphorylation of MEK1/2 by serum (A) or PDB (B) in vascular smooth muscle cells. Quiescent vascular smooth muscle cells were incubated with or without pentalenolactone at the indicated concentration for 4 h and then stimulated briefly with serum (5 min at 37°C) or PDB (10 min at 37°C). Phosphorylation and total expression of MEK1/2 were determined by Western blot analysis using anti-phospho-MEK1/2 and anti-MEK1/2 antibodies, respectively. p-MEK and MEK indicate the phosphorylated form of MEK and total MEK, respectively. Similar results were obtained in two (A) or six (B) separate experiments.

3.6. Effect of pentalenolactone on tyrosine phosphorylation of the PDGF receptor

As many studies have suggested that PDGF is a potent mitogen for vascular smooth muscle cells, and that phosphorylation of the PDGF receptor is part of the early signaling cascade of the mitogen (Majesky et al., 1990; Casscells, 1991; Thyberg et al., 1990), we attempted to clarify the effect of pentalenolactone on autophosphorylation of the PDGF receptor. When quiescent vascular smooth muscle cells were stimulated with 30 ng/ml PDGF-BB for 1 min, marked tyrosine-phosphorylation of the protein band at 180 kDa was observed (Fig. 6). Since it is known that PDGF β-receptor binds only the PDGF-B chain (LaRochelle et al., 1991; Claesson-Welsh, 1994), this protein band was thought to be that of the PDGF β-receptors. This tyrosine phosphorylation was inhibited by pretreatment of cells with tyrphostin AG 1295 (10 μM), a selective inhibitor of PDGF receptor autophosphorylation (Fig. 6) (Kovalenko et al., 1994). Pentalenolactone was less effective on PDGF receptor autophosphorylation (Fig. 6).

3.7. Effects of pentalenolactone on Swiss 3T3 cell proliferation, motility and glycolysis

Pentalenolactone was originally thought to inhibit glycolysis by inhibition of GAPDH. In the next series of experiments, we compared the efficacy of the inhibitory effects of pentalenolactone on cell proliferation and glycolysis in Swiss 3T3 cells, because cellular glycolysis of vascular smooth muscle cell, as determined from the lactate content of the conditioned medium had been hardly detectable under our experimental condition. We found that pentalenolactone inhibited proliferation of Swiss 3T3 cells dose-dependently when added in the logarithmic growth phase, with an IC_{50} value of 0.74 μM (from four independent experiments). Cellular glycolysis was decreased by pentalenolactone dose-dependently with an IC_{50} of 7.4 μM (from three independent experiments). On the other hand, cytotoxicity of pentalenolactone in Swiss 3T3 cells, which was determined by MTT assay in cells of

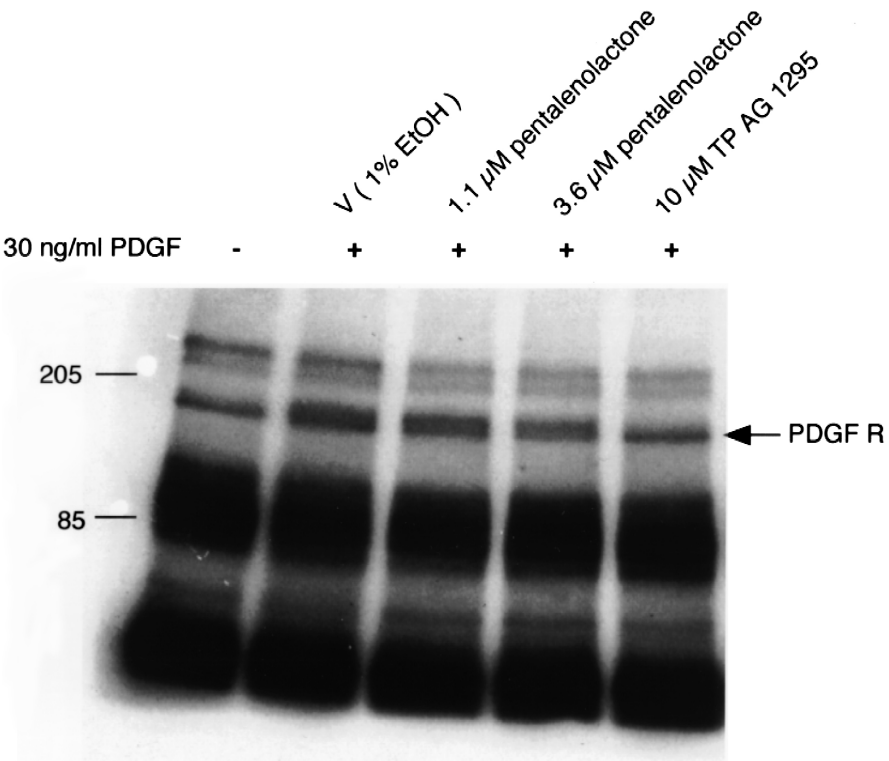

Fig. 6. Effect of pentalenolactone on autophosphorylation of the PDGF receptor in vascular smooth muscle cells. Quiescent vascular smooth muscle cells were incubated with pentalenolactone or tyrphostin AG 1295 at the indicated concentration, or without drug for 4 h. Cells were then stimulated briefly with PDGF-BB (1 min at 37°C). Extracts were subjected to SDS-PAGE in a 7.5% acrylamide gel and immunoblotting with anti-phosphotyrosine antibody. The numbers on the left indicate the molecular mass in kiloDaltons. PDGF R indicates the localization of the PDGF receptor. Similar results were obtained in three separate experiments.

saturated density, was observed only at a high concentration. The viability of quiescent 3T3 cells was decreased by 50% at 260 μM (from four independent experiments).

4. Discussion

In vascular smooth muscle cells, it has been reported that various mitogens including angiotensin II, PDGF-BB, insulin and thrombin, activate the ERK1/2 pathway at early in the G1 phase along with cell growth, and that inhibition of the pathway by PD98059, an inhibitor of MEK1/2, blocks mitogen-induced cellular responses (Bornfeldt et al., 1997; Xi et al., 1997, 1999; Weiss et al., 1998). Therefore, the ERK pathway is thought to play an important role in early mitogenic signal transduction by vascular smooth muscle cells as well as in other cell types.

In the present study, pentalenolactone was shown to inhibit rat vascular smooth muscle cell proliferation and DNA synthesis in response to serum. The inhibitory effect of pentalenolactone on DNA synthesis was attenuated even when added 2 h after stimulation with serum, and the inhibitory effect of pentalenolactone on incorporation of BrdU into the cells gradually declined as the delay between treatment of vascular smooth muscle cells with pentalenolactone and serum increased. Similar inhibitory effect was also observed with PD98059, a MEK inhibitor. The restriction point and G1/S boundary for the vascular smooth muscle cells were estimated to be around 8 and 16 h after stimulation with serum, respectively. These data suggest that pentalenolactone inhibits cell proliferation by arresting the cell cycle in G1 phase, and that this effect is mediated by inhibition of an early G1 event during mitogenic signal transduction. Furthermore, we observed that pentalenolactone inhibited both serum-and PDB-induced phosphorylation of ERK1/2 and MEK1/2. On the basis of these results, one possible mechanism for the antiproliferative effect of pentalenolactone in vascular smooth muscle cells may be inhibition of activation of the MEK–ERK cascade.

Pentalenolactone has been shown to inhibit GAPDH in mammalian cells, as well as in a wide variety of microorganisms (Hartmann et al., 1978; Mann and Mecke, 1979; Duszenko et al., 1982; Lambeir et al., 1991; Cane and Sohng, 1989). As inhibition of GAPDH leads to depletion of cellular ATP, it is possible that this depletion results in inhibitions of serum- and PDB-induced phosphorylation of ERK1/2 and MEK1/2. However, we showed that pental-

enolactone at a dose causing maximal inhibition of ERK1/2 and MEK1/2 phosphorylation, had little effect on PDGF-BB-induced autophosphorylation, suggesting that ATP depletion by pentalenolactone treatment was minimal, if any. Furthermore, pentalenolactone inhibited glycolysis in Swiss 3T3 cells with an IC50 value of 7.4 μM, which was 10 times higher than that necessary for Swiss 3T3 cell proliferation. It has also been reported that measurable inhibition of mammalian cell glycolysis is detectable at a pentalenolactone dose of 7 μM or more (Duszenko et al., 1982). These suggested that the mechanism underlying the inhibition of cell proliferation by pentalenolactone was independent of GAPDH inhibition. Therefore, it is likely that the inhibitory effects of pentalenolactone on serum- and PDB-induced ERK1/2 and MEK1/2 phosphorylation are not mediated by GAPDH inhibition.

It has been suggested that activation of protein kinase C results in phosphorylation of ERK1/2 through the c-Raf1-MEK cascade (Meloche et al., 1992a,b). Moreover, membrane-associated intracellular conventional protein kinase C activity has been shown to increase following stimulation of cultured rat vascular smooth muscle cells with serum (Ottlinger et al., 1993; Herbert et al., 1996). In the present study, pentalenolactone was shown to inhibit the phosphorylations of ERK1/2 and MEK1/2 in response to serum and PDB, which activated conventional/novel protein kinase C (Lee and Severson, 1994). Therefore, the target site of pentalenolactone appears to lie between protein kinase C and MEK1/2. In order to explore the exact target site of pentalenolactone, further studies are required, including an investigation of the effect of pentalenolactone on protein kinase C, c-raf-1 and MEK of vascular smooth muscle cells.

In summary, we have demonstrated for the first time that the sesquiterpenoid pentalenolactone has inhibitory effects against vascular smooth muscle cell proliferation, and that its mechanism of action appears to be mediated, at least in part, by inhibition of ERK1/2 activation.

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