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Antiproliferative effect in rat vascular smooth muscle cells by osthole, isolated from *Angelica pubescens*

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Abstract

The antiproliferative effect of osthole on rat vascular smooth muscle cells was examined in this study. A number of mitogenic agents, e.g., foetal-calf serum (10%, v/v) and platelet-derived growth factor (20 ng/ml), and pharmacological agents, e.g., serotonin (10 μM), ionomycin (3 nM), phorbol 12,13-dibutyrate (20 nM) and phorbol myristate acetate (200 nM), were used to induce DNA synthesis in rat vascular smooth muscle cells; these effects were concentration dependently inhibited by osthole and the half-maximal inhibition (IC_{50}) occurred at 13.6 ± 1.8, 11.8 ± 1.3, 7.9 ± 0.9, 7.1 ± 0.2, 7.8 ± 0.2 and 8.6 ± 0.4 μM, respectively. Osthole itself increased the cyclic AMP and cyclic GMP formations in a concentration-dependent manner; it synergistically increased cyclic AMP and cyclic GMP levels induced by forskolin and sodium nitroprusside, respectively. After 48 h deprivation of serum, cells were re-stimulated with serum and the cell cycle was observed by flow cytometry; treatment of cells with osthole (100 μM) caused a block of serum-inducible cell cycle progression at a point before the G_1–S boundary. The addition of osthole (100 μM) at various times after serum addition to serum-deprived cells showed full inhibition of DNA synthesis even when added 6 h after serum. The cell cycle progression block was gradually lost as the delay from serum to osthole application was increased from 6 to 18 h. The effect of osthole on serum-stimulated [^{3}H]thymidine incorporation into endothelial cells was examined and the IC_{50} value (158.7 ± 2.7 μM, $n = 6$) was obtained; it exhibited greater potency (12-fold) for vascular smooth muscle cells as compared with endothelial cells as an antiproliferative agent. These results suggest that osthole is a selective antiproliferative agent in vascular smooth muscle cells. The antiproliferative effect occurs at the early G_1 phase of the cell cycle and is due to the increase in cyclic AMP and cyclic GMP contents.

Keywords: DNA synthesis; Cell cycle; Smooth muscle cell, rat, vascular; Osthole; (*Angelica pubescens*)

1. Introduction

Abnormal vascular smooth muscle cell proliferation is a major component of vascular disease, including atherosclerosis, vein graft occlusion and re-stenosis following angioplasty (Ross, 1986; Ip et al., 1990). Intimal vascular smooth muscle cell proliferation in vascular disease is thought to result from endothelial dysfunction or injury (Campbell and Campbell, 1985). In a number of studies, growth inhibition of smooth muscle cells, when in coculture with endothelial cells, has been ascribed to the actions of endothelium-derived heparin/heparan sulphate proteoglycans (Scott-Burden and Buhler, 1988) and of the elevation of intracellular cyclic GMP in smooth muscle cells (Scott-Burden and Vanhoutte, 1993). An important therapeutic aim is to inhibit vascular smooth muscle cell proliferation without interfering with endothelial repairs.

A number of vasoconstrictors, e.g., angiotensin II, serotonin and endothelin, are capable of stimulating vascular smooth muscle cell proliferation (Uehara et al., 1991; Daemen et al., 1991; Bobik et al., 1990). In cultured vascular smooth muscle cells, they activate many of the same cellular responses as mitogens such as platelet-derived growth factor (PDGF) do, including stimulation of phospholipase C (Smith, 1986; Kawahara et al., 1988), mobilization of intracellular Ca^{2+} (Alexander et al., 1985; Brock et al., 1985) and stimulation of expression of c-*fos* and c-*myc* (Taubman et al., 1989; Naftilan et al., 1989; Bobik et al., 1990). Additionally, PDGF and epidermal growth factor increase tension in aortic strips (Berk et al.,

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1985; Berk et al., 1986), thereby sharing vasoconstrictor properties with those of pharmacological vasoconstrictors. Furthermore, the cyclic nucleotides, cyclic AMP and cyclic GMP, have been reported to inhibit the proliferation of several cell types (Friedman, 1976; Garg and Hassid, 1989); several Ca^{2+} antagonists (nifedipine, verapamil and diltiazem) have also been reported to inhibit vascular smooth muscle cell proliferation in animal models (Jackson et al., 1988). This implies that there is coordinated control of acute vascular smooth muscle cell function and long-term development.

In our previous studies, we have found that osthole, an active principle isolated from dried root of *Angelica pubescens* by hot methanol extraction and a series of purification methods, exerts relaxant action in rat thoracic aorta and guinea-pig tracheal smooth muscle (Ko et al., 1992; Teng et al., 1994). In this study, the antiproliferative effect and the action mechanism of osthole in rat vascular smooth muscle cells are investigated. Additionally, the selectivity of its antiproliferative effect is also evaluated.

2. Materials and methods

2.1. Cell culture

The smooth muscle cell line, A10, derived from the rat thoracic aorta was provided by American Type Cell Collection. Cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% foetal-calf serum, 100 units/ml penicillin G and 100 μg/ml streptomycin sulphate at 37°C in a humidified, 5% CO_2-in-air atmosphere. To subculture the cells, confluent monolayers were washed with phosphate-buffered saline (PBS); treated with 0.1% trypsin-0.04% EDTA, placed in an equal volume of medium, and centrifuged at $600 \times g$ for 5 min. Cells were seeded into T-75 flasks at an initial density of 2×10^4 cells/cm^2.

2.2. Assay of [^{3}H]thymidine incorporation

For the assay of agonist-induced DNA synthesis, cells were grown to confluence in 24-well tissue culture plates. Subcultured cells were made quiescent in serum-free medium for 48 h before re-stimulation by the addition of 10% foetal-calf serum, PDGF-BB or pharmacological agents (timed as 0 h for all subcultured cell experiments).

Osthole or cyclic nucleotide-elevating agents were added at various concentrations to cells on plating out at 0 h. [^{3}H]Thymidine was added to cells 18 h after the addition of agonists at a concentration of 1 μCi/ml and was incubated for a further 6 h. At the end of the incubation, radioactive medium was removed, and cells were washed with PBS before processing for determination of the levels of [^{3}H]thymidine incorporated into DNA.

2.3. Assay of cyclic AMP and cyclic GMP contents

Confluent and quiescent cultures were washed 3 times at 37°C and then incubated in 1 ml DMEM in the presence of osthole, forskolin, sodium nitroprusside or 3-isobutyl-1-methylxanthine (IBMX) for 10 min. At the end of the specified incubation period, the medium was rapidly removed and 0.5 ml ice-cold 10% (w/v) trichloroacetic acid-4 mM EDTA was added into the wells. After incubation for 30 min at 4°C, cells were scraped and the resulting suspensions were centrifuged at $10000 \times g$ for 5 min and the supernatants were removed and extracted with 4×3 volumes of ether. Next, the cyclic AMP and cyclic GMP contents were then assayed using enzyme-immunoassay (EIA) kits. Pellets were resuspended in PBS buffer for protein assay.

2.4. Assay of [^{3}H]inositol monophosphate accumulation

Cells for inositol phospholipid hydrolysis were finally grown in 12-well plates. Monolayer cells were made quiescent for 24 h and were then loaded with [^{3}H]myo-inositol (5 μCi/ml) for a further 24 h in serum-free DMEM. Prelabelled cells were then washed twice with serum-free DMEM and incubated for 15 min in the presence of 10 mM LiCl. Foetal-calf serum (10%, v/v) was added and incubation continued for 30 min. Incubation was terminated by a stopping buffer and the addition of 0.1 N HCl (1 : 1 v/v). Cells were left a minimum of 30 min at −20°C before isolation of total [^{3}H]inositol phosphates by anion exchange chromatography (Alexander et al., 1989). Briefly, 800 μl of supernatant was neutralized by addition of 300 μl of 0.1 N NaOH, 1 ml of 50 mM Tris-HCl and added to an AG1-X8 column. The resin was washed with a total of 25 ml H_2O. The [^{3}H]inositol monophosphate was then eluted with 3 ml of 0.2 M ammonium formate/0.1 M formic acid. Radioactivity was determined by scintillation counting after the addition of scintillation cocktail.

2.5. Flow cytometric assay

Cells were plated to attach, and placed in serum-free medium for 48 h as described above. Dimethylsulphoxide (DMSO, 0.1%) or osthole (100 μM) was added in the serum-free medium followed by 10% foetal-calf serum-containing medium. At the specified times after serum addition, these cells were harvested by PBS/EDTA, washed 2 times with PBS and fixed in 70% (v/v) ethanol at 4°C for 30 min. After fixation, cells were centrifuged and resuspended in PBS. The PBS contained 40 μg/ml propidium iodide and 0.1 mg/ml RNase. After 30 min at 37°C, cells were analyzed on a FACStar cytofluorometer (Becton-Dickinson; San Jose, CA, USA) equipped with an argon-ion laser at 488 nm.

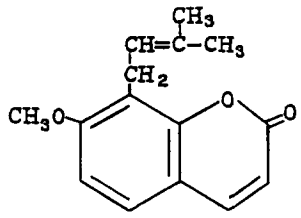

Fig. 1. Chemical structure of osthole.

2.6. Data analysis

The results are expressed as means ± S.E.M. for the indicated number of separate experiments. Statistical significance between drug-treated and untreated groups was evaluated by Student's *t*-test and *P* values of less than 0.05 were considered significant.

2.7. Materials

Osthole (Fig. 1) was isolated from *Angelica pubescens* as described previously (Wu et al., 1990). The following materials were used: DMEM and all other tissue culture reagents were obtained from GIBCO (Grand Island, NY, USA). [^{3}H]Thymidine and myo-[2-^{3}H]inositol (10–20 Ci/mmol) were purchased from Amersham (Buckinghamshire, UK). Human PDGF-BB was purchased from Boehringer Mannheim (Mannheim, Germany). Ionomycin, phorbol 12,13-dibutyrate (PDB), phorbol 12-myristate 13-acetate (PMA), propidium iodide, RNase, sodium nitroprusside, forskolin, IBMX, 8-bromo-cyclic AMP, 8-bromo-cyclic GMP and serotonin were obtained from Sigma Chem. Co., USA. Cyclic AMP and cyclic GMP EIA kits were purchased from Cayman Chem. Co., USA and AG1-X8 (100–200 mesh) resin was purchased from Bio-Rad Lab. If drugs were dissolved in DMSO, the final concentration of DMSO in the bathing solution did not exceed 0.1% and exerted no effect on the cultured cells.

3. Results

3.1. Effect of osthole and various antiproliferative agents on serum-stimulated thymidine incorporation

The actions of osthole and some antiproliferative agents on foetal-calf serum (10%, v/v)-stimulated thymidine incorporation into quiescent cultured vascular smooth muscle cells were evaluated; they all concentration dependently inhibited the incorporation of thymidine (Fig. 2). Sodium nitroprusside (IC_{50} = 11.0 ± 0.9 μM) and osthole (IC_{50} = 13.6 ± 1.8 μM) were the most potent followed by forskolin (IC_{50} = 33.1 ± 1.4 μM), 8-bromo-cyclic GMP (IC_{50} = 171.4 ± 7.8 μM) and 8-bromo-cyclic AMP (IC_{50} = 553.0 ± 11.2 μM). PDGF-BB (20 ng/ml) and serotonin (10 μM) also stimulated thymidine incorporation into rat vascular smooth muscle cells; these effects were inhibited by osthole (1–30 μM) and the IC_{50} values were 11.8 ± 1.3 and 7.9 ± 0.9 μM, respectively.

3.2. Effect of osthole on ionomycin-, PDB- and PMA-stimulated proliferation

Ca^{2+} ionophore, ionomycin, and protein kinase C activators, PDB and PMA, are capable of inducing mitogenesis. Addition of these mitogenic agents to quiescent vascular smooth muscle cells led to the elevation of DNA synthesis; these actions were concentration-dependent (data not shown) and the submaximal concentrations were selected. Osthole concentration dependently (3–30 μM) inhibited ionomycin (3 nM), PDB (20 nM) and PMA (200 nM)-stimulated thymidine incorporation (Fig. 3); the IC_{50} values were 7.1 ± 0.2, 7.8 ± 0.2 and 8.6 ± 0.4 μM, respectively. In addition, nifedipine was tested in ionomycin (3 nM)-stimulated thymidine incorporation; only a high concentration (30 μM) of nifedipine significantly inhibited this action (Fig. 3B).

3.3. Effect of osthole on cyclic nucleotide formation and serum-induced phosphoinositide breakdown

The effect of osthole on cyclic nucleotide formation was measured in rat vascular smooth muscle cells. As shown in Table 1, osthole (10–100 μM) caused a concentration-dependent increase in cyclic AMP and cyclic GMP contents. The cyclic AMP and cyclic GMP levels were also elevated by forskolin (3 μM) and sodium nitroprusside (1 μM), respectively. IBMX, a non-selective phosphodiesterase inhibitor, caused a marked increase in

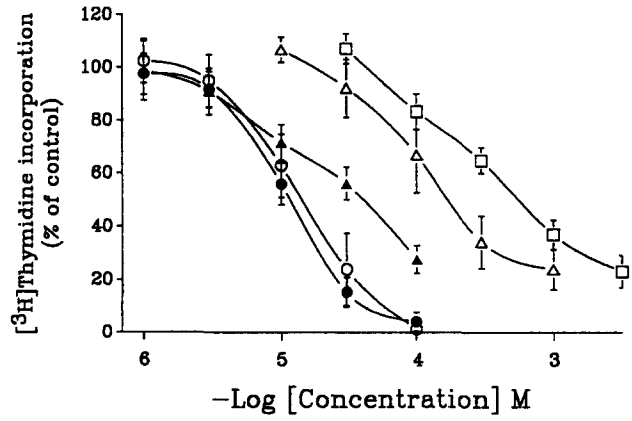

Fig. 2. Effects of osthole and antiproliferative agents on serum-stimulated DNA synthesis. Quiescent rat vascular smooth muscle cells were incubated in quadruplicate for 24 h with 10% (v/v) foetal-calf serum and the concentrations shown of osthole (○), sodium nitroprusside (●), forskolin (▲), 8-bromo-cyclic AMP (□) or 8-bromo-cyclic GMP (△) were added to inhibit foetal-calf serum-stimulated DNA synthesis. DNA synthesis assayed by [^{3}H]thymidine incorporation as described in Materials and methods is expressed relative to that of control cells to which only serum was added. Values are means ± S.E.M. of six determinations.

cyclic AMP and cyclic GMP levels. In addition, the combination of IBMX (100 μM) or osthole (100 μM) with forskolin (3 μM) or with sodium nitroprusside (1 μM) produced a synergistic increase in cyclic AMP or cyclic GMP contents, respectively. However, the combination of osthole (100 μM) with IBMX (100 μM) did not further increase cyclic nucleotide content compared with each agent alone (Table 1). The effect of osthole on serum-stimulated phosphoinositide breakdown was also measured. Rat vascular smooth muscle cells were labelled with [^{3}H]myo-inositol; the accumulation of [^{3}H]inositol monophosphate was increased in the presence of 10% foetal-calf serum. Osthole (10–100 μM) had no influence on this effect (data not shown).

3.4. Effect of osthole on serum-stimulated cell cycle

To determine when in the cell cycle osthole exerted its inhibitory effect on proliferation, subcultured vascular smooth muscle cells were made quiescent by serum deprivation for 48 h, and approximately 85% of cells had a 2n DNA complement, consistent with the location in the G_0/G_1 phase of the cell cycle. Following replacement of serum-free medium with DMEM containing 10% foetal-calf serum, the emergence of cells into and through the *S* phase was observed by flow cytometry using quantitative DNA staining with propidium iodide (Fig. 4A). The majority of cycling cells was observed to progress through the *S* phase during an interval from approximately 12–18 h after serum repletion. In the presence of osthole (100 μM), however, the majority of cycling cells remained in the G_0/G_1 phase (Fig. 4B). In the other experiment, subcultured cells were made quiescent by serum deprivation for 48 h before re-stimulation with serum, and thymidine incorporation was assayed after 24 h. Full inhibition of DNA synthesis only occurred when osthole (100 μM) was present within the first 6 h after the re-addition of serum, and there was no significant effect on DNA synthesis when osthole was added 15 h after the serum refeeding (Fig. 5).

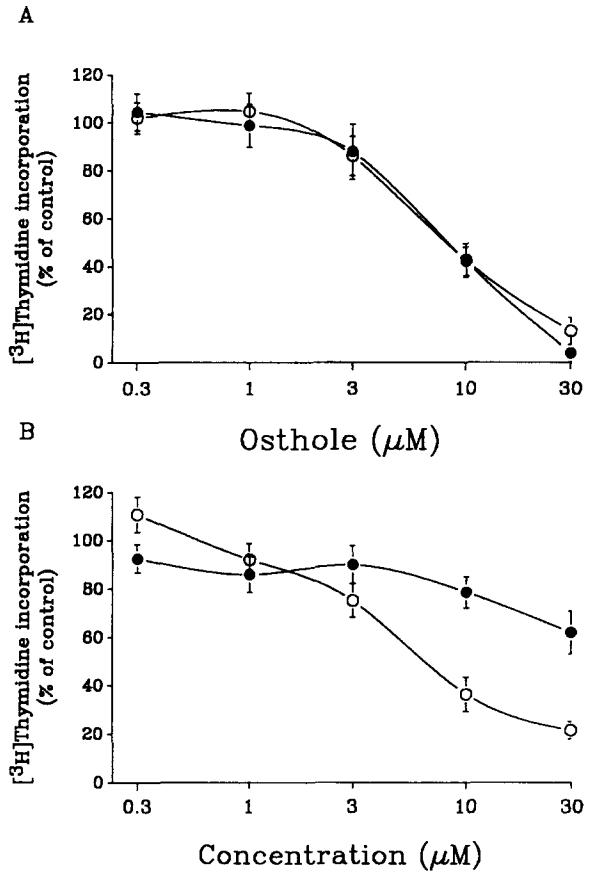

Fig. 3. Effects of osthole on (A) PDB- and PMA- or (B) ionomycin-stimulated DNA synthesis. Quiescent rat vascular smooth muscle cells were incubated in quadruplicate for 24 h with PDB (○, 20 nM), PMA (●, 200 nM) or ionomycin (3 nM) and the concentrations shown of osthole (○ and ●, for A and ○, for B) or nifedipine (●, for B) were added to inhibit DNA synthesis stimulated by these agents. DNA synthesis assayed by [^{3}H]thymidine incorporation as described in Materials and methods is expressed relative to that of control cells to which only agonists were added. Values are means ± S.E.M. of six determinations.

Table 1
Effects of osthole, forskolin, sodium nitroprusside and 3-isobutyl-1-methylxanthine (IBMX) on the cyclic AMP and cyclic GMP formations of rat vascular smooth muscle cells

Addition	Cyclic AMP (pmol/mg protein)	Cyclic GMP (pmol/mg protein)
Control	1.9 ± 0.6	3.7 ± 0.9
Forskolin 3 μM	30.2 ± 2.9 [b]	
Sodium nitroprusside 1 μM		7.8 ± 0.8 [a]
Osthole 10 μM	5.2 ± 0.8 [a]	6.2 ± 0.9
30 μM	8.3 ± 1.2 [b]	9.6 ± 1.0 [a]
100 μM	23.2 ± 2.1 [b]	14.8 ± 1.2 [b]
Osthole 100 μM + forskolin 3 μM	97.8 ± 3.2 [b]	
Osthole 100 μM + sodium nitroprusside 1 μM		39.7 ± 3.1 [b]
IBMX 100 μM	19.8 ± 2.7 [b]	15.7 ± 1.1 [b]
IBMX 100 μM + forskolin 3 μM	89.2 ± 1.7 [b]	
IBMX 100 μM + sodium nitroprusside 1 μM		41.2 ± 2.3 [b]
Osthole 100 μM + IBMX 100 μM	25.8 ± 2.0 [b]	19.4 ± 0.9 [b]

After preincubation of rat vascular smooth muscle cells in 1 ml DMEM for 5 min, dimethylsulphoxide (0.1%, control), osthole, forskolin, sodium nitroprusside or IBMX of the indicated concentrations was added for another 10 min and the reaction was stopped by the addition of ice-cold 10% (w/v) trichloroacetic acid/4 mM EDTA as described in Methods. Cyclic AMP and cyclic GMP contents were then measured. Values are means ± S.E.M. of three determinations. [a] $P < 0.01$ and [b] $P < 0.001$ as compared with the respective control.

3.5. Effect of osthole on serum-stimulated endothelial cell proliferation

The antiproliferative effect of osthole to bovine pulmonary endothelial cells was examined. Endothelial cells

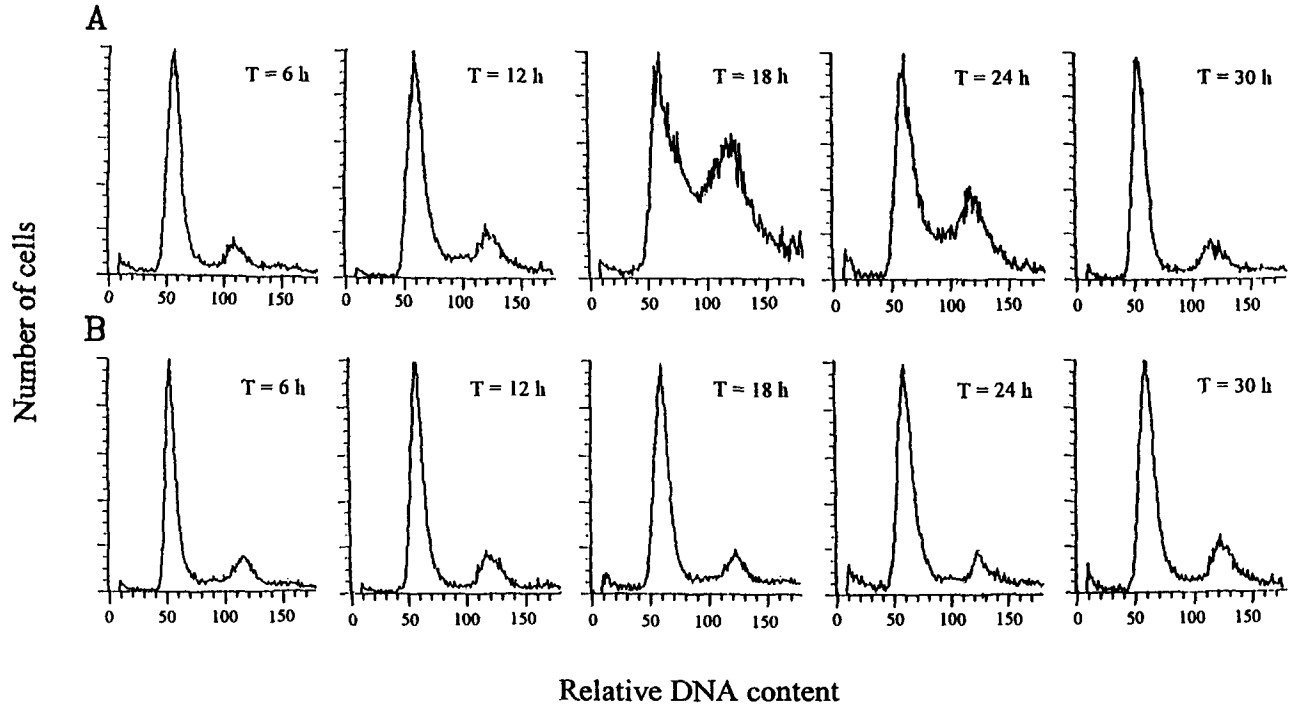

Fig. 4. Time dependence of cell cycle progression as evaluated by flow cytometric determination of DNA content in quiescent rat vascular smooth muscle cells in the absence (A) or presence (B) of osthole (100 μM). Times given are those transpiring between 10% (v/v) foetal-calf serum repletion and cell harvesting as observed by flow cytometry. Individual nuclear DNA content as reflected by fluorescence intensity of incorporated propidium iodide is plotted as a histogram depicting relative cell numbers at each intensity. Each histogram is derived from a data collection of 10000 events and is representative of histograms obtained from a multiple of such experiments; the depicted histograms were those obtained within one experiment.

were made quiescent by serum deprivation for 48 h before re-stimulation with serum and then osthole was added to inhibit foetal-calf serum (10%, v/v)-stimulated thymidine incorporation and the IC_{50} value was 158.7 ± 2.7 μM ($n = 6$).

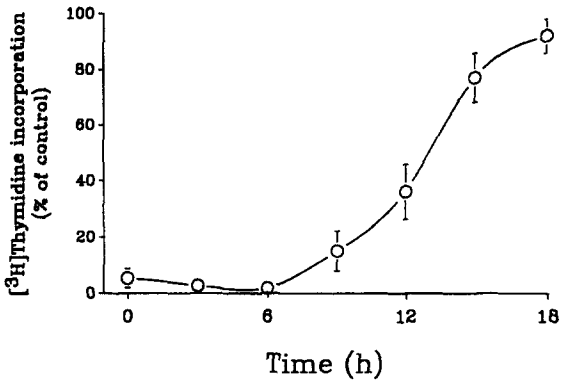

Fig. 5. Effect of delayed addition of osthole on DNA synthesis. Subcultured rat vascular smooth muscle cells were made quiescent by serum deprivation for 48 h. Cells were exposed to osthole (100 μM) at the indicated times after the addition of 10% (v/v) foetal-calf serum. DNA synthesis was assayed by [^{3}H]thymidine incorporation from 18 to 24 h. DNA synthesis is expressed relative to that of control cells to which no osthole was added. Values are means ± S.E.M. of four determinations (each in quadruplicate).

4. Discussion

Vascular smooth muscle cells cease to proliferate when they are made quiescent by deprivation of serum. Such quiescent cells can be stimulated to reinitiate DNA synthesis and cell division either by replenishing the medium with fresh serum, or by the addition of growth factors or pharmacological agents in serum-free medium (Newby and George, 1993). In this study, serum, PDGF-BB and serotonin stimulated DNA synthesis in rat vascular smooth muscle cells; osthole inhibited these effects in a concentration-dependent manner and had similar IC_{50} values. In previous studies, the cyclic nucleotide-elevating agents were proved to be capable of inhibiting the proliferation of several cell types, including rat and rabbit vascular smooth muscle cells (Friedman, 1976; Assender et al., 1992; Garg and Hassid, 1989). In the present study, forskolin, 8-bromo-cyclic AMP, sodium nitroprusside and 8-bromo-cycilc GMP concentration dependently inhibited [^{3}H]-thymidine incorporation into rat vascular smooth muscle cells. Sodium nitroprusside and osthole were the most potent and had similar IC_{50} values.

The primary proliferative signals required for DNA synthesis in vascular smooth muscle cells have not been defined. Many of the mitogenic agents, e.g., serum, growth factors and pharmacological agents, are known to generate the primary signal: increased phosphoinositide breakdown

(Smith, 1986; Kawahara et al., 1988). Increased phosphoinositide breakdown and the latter events, protein kinase C activation and Ca^{2+} mobilization, are therefore deemed necessary for subsequent DNA synthesis. In this study, serum stimulated phosphoinositide breakdown in rat vascular smooth muscle cells; osthole (10–100 μM) had no inhibition on this effect. We assumed that osthole blocked the events after phospholipase C activation to inhibit vascular smooth muscle cell proliferation.

Protein kinase C activators are reported to be capable of stimulating proliferation of several cell types (Dicker and Rozengurt, 1980; Collins and Rozengurt, 1982; Wang et al., 1992) and it is suggested that Ca^{2+} mobilization and protein kinase C activation act synergistically to maximally induce cell proliferation. Since activation of protein kinase C and stimulation of Ca^{2+} mobilization may play varying roles in stimulating proliferation, examining the effect of osthole on these events is crucial. In this study, ionomycin (3 nM), the Ca^{2+}-mobilizing agent, and PDB (20 nM) and PMA (200 nM), the protein kinase C activators, stimulated [^{3}H]thymidine incorporation into rat vascular smooth muscle cells. Osthole concentration dependently inhibited the DNA synthesis stimulated by these agents and showed similar IC_{50} values. It is suggested that the observed osthole effects are related, at least partially, to effects on Ca^{2+} mobilization- and protein kinase C-mediated pathways.

In our previous studies, osthole was shown to exert relaxant effects on rat thoracic aorta and guinea-pig trachea; these effects were due to the inhibition of Ca^{2+} influx and of cyclic AMP and cyclic GMP phosphodiesterases, respectively (Ko et al., 1992; Teng et al., 1994). In this study, osthole concentration dependently inhibited ionomycin-stimulated DNA synthesis. However, nifedipine was ineffective towards this action except when a high concentration (30 μM) was used. This implies that the antiproliferative effect of osthole is not due to the inhibition of extracellular Ca^{2+} influx from voltage-operated Ca^{2+} channels. The formation in cyclic AMP and cyclic GMP was measured in rat vascular smooth muscle cells. Osthole increased cyclic AMP and cyclic GMP contents in a concentration-dependent manner. The combination of osthole with forskolin or with sodium nitroprusside produced a synergistic increase in cyclic AMP or cyclic GMP content, respectively. The combination of osthole with IBMX did not further increase these cyclic nucleotide contents compared with each agent alone. It is suggested that osthole is a phosphodiesterase inhibitor and the antiproliferative effect of osthole is due to the increase in cyclic AMP and cyclic GMP formation.

To determine when in the cell cycle osthole exerted its inhibitory effect on proliferation, rat vascular smooth muscle cells were synchronized to the quiescent state (G_0) by serum deprivation and then stimulated to enter the cell cycle by serum refeeding. In the absence of osthole, a typical cell cycle with the successive phases was obtained by flow cytometry using quantitative DNA staining with propidium iodide. In the presence of osthole (100 μM), however, the majority of cycling cells remained in the G_0/G_1 phase. Further definition of the inhibition point of the cell cycle by osthole was obtained from experiments in which the time of osthole (100 μM) exposure was varied relative to the time of serum stimulation. The results indicated that full inhibition of DNA synthesis only occurred when osthole was present within the first 6 h after the re-addition of serum. These data imply that osthole inhibits early events in the G_1 phase of the cell cycle.

When the vascular endothelium is damaged, the underlying vascular smooth muscle cells are exposed to a variety of potential mitogenic agents to induce vascular smooth muscle cell proliferation. Agents which selectively inhibited vascular smooth muscle cell proliferation without affecting endothelial repair might be expected. Indeed, endothelial cells of different origins present different phenotypes and different responses to modulators. In this study, bovine pulmonary endothelial cells were used to examine the selectivity of the antiproliferative effect of osthole. The results showed that 10% foetal-calf serum significantly stimulated DNA synthesis of bovine pulmonary endothelial cells. Osthole concentration dependently inhibited this effect with an IC_{50} value of 158.7 $\pm$ 2.7 μM; it exhibited greater potency (12-fold) as an antiproliferative agent for rat vascular smooth muscle cells as compared with bovine pulmonary endothelial cells.

In conclusion, osthole selectively inhibited rat vascular smooth muscle cell proliferation. The antiproliferative effects of osthole occur at the early G_1 phase of the cell cycle and are due to the increase in cyclic AMP and cyclic GMP contents.

Acknowledgements

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