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Effect of *S*(−) perillic acid on protein prenylation and arterial smooth muscle cell proliferation☆

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Abstract

A number of proteins post-translationally modified by the covalent attachment of mevalonate-derived isoprene groups farnesol (FOH) or geranylgeraniol (GGOH), play a role in cell proliferation. For this reason, protein farnesyltransferase (PFTase) and protein geranylgeranyltransferases (PGGTases) I and II have gained attention as novel targets for the development of antiproliferative agents. Monoterpenes [limonene, perillic acid (PA) and its derivatives] have been shown to inhibit cell growth and protein prenylation in cancer cells. In the present study, we evaluated the effect of *S*(−) PA on diploid rat aorta smooth muscle cell (SMC) proliferation as related to protein prenylation. *S*(−) PA (1–3.5 mM) decreased, in a concentration-dependent manner, rat SMC proliferation as evaluated by cell counting and DNA synthesis. Morphological criteria and flow cytometry analysis excluded the induction of apoptosis as a potential antiproliferative mechanism of *S*(−) PA on SMC and confirmed a block of the cell cycle progression in G_0/G_1 phase. The antiproliferative effect of *S*(−) PA could not be prevented by the addition of mevalonate, FOH, and GGOH to the culture medium and was independent of cholesterol biosynthesis. Densitometric analysis of fluorographed gels, after sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the cell lysates, further supported that *S*(−) PA (1–3.5 mM), under the same experimental conditions, concentration-dependently inhibited FOH (up to 70%) and GGOH (up to 70%) incorporation into cellular proteins. We provide evidence that *S*(−) PA affects protein prenylation, an effect that may contribute to its inhibition of SMC proliferation.

Keywords: Isoprenoids; Atherosclerosis; Farnesol; Geranylgeraniol; Farnesyltransferase inhibitors; Geranylgeranyltransferase inhibitors

1. Introduction

Smooth muscle cell (SMC) proliferation in the arterial wall is a prominent feature of atherogenesis and a possible determinant of restenosis after angioplasty [1]. Thus, the elucidation of the factors affecting these phenomena affords new entry points for selective pharmacological interference in these processes. Mevalonate (MVA) and its derivatives (isoprenoids) are essential for cell growth [2,3]; hence, drugs affecting this metabolic pathway, such as 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), may reduce cell proliferation. Our observation that two MVA-derived prenyl alcohols, all-*trans* farnesol (FOH) and all-*trans* geranylgeraniol (GGOH), can prevent statin-induced inhibition of SMC growth, in the absence of other MVA-derived isoprenoids [4,5], together with studies showing that both all-*trans* FOH and all-*trans* GGOH are readily incorporated into cellular proteins [6,7], supports the potential role of prenylated protein(s) in the control of SMC proliferation.

Several proteins post-translationally modified by the covalent attachment of the MVA-derived isoprenoid groups farnesyl and geranylgeranyl have been identified [3,8].

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Abbreviations: FOH, farnesol; GGOH, geranylgeraniol; PFTase, protein farnesyltransferase; PGGTase, protein geranylgeranyltransferase; PA, perillic acid; SMC, smooth muscle cell; MVA, mevalonate; HMG-CoA, 3-hydroxy-3-methyl-glutaryl coenzyme A; FCS, fetal calf serum; MEM, Minimum Essential Medium; PBS, phosphate buffered saline; PI, propidium iodide.

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These proteins must be prenylated as a prerequisite for membrane association, which, in turn, is required for their function [3]. For this reason, the three classes of enzymes that catalyze the addition of prenyl groups to proteins, protein farnesyltransferase (PFTase) and protein geranylgeranyltransferases (PGGTase I and II) [9,10], have gained attention as novel targets for the development of agents aimed at controlling abnormal cell growth, such as SMC proliferation under atherogenic condition or tumor development [1].

Among the prenylated proteins, much attention has been focused on p21ras for its key role in the pathophysiology of cell proliferation [8]. Because farnesylation of p21ras is required for its mitogenic activity, an intensive search and development for PFTase inhibitors is underway [11]. Several classes of PFTase inhibitors with antiproliferative activity in cell cultures and in animal models have been described, and subsets are undergoing clinical trials. However, the demonstration that K_B-ras [12], rho b [3], as well as some 25 unidentified proteins [7] can be either farnesylated or geranylgeranylated has led to an interest in developing the combination of PFTase and PGGTase inhibitors or double inhibitors of PFTase and PGGTase [13]. Among these double inhibitors are a series of monoterpenes, including limonene, perillyl alcohol, and their *in vivo* metabolites, which inhibits both PFTase and PGGTases I and II [14–16] and, as a result, protein farnesylation and geranylgeranylation in cultured cells [15,17–21]. In addition, the naturally occurring monoterpenes *R*(+) limonene and *S*(−) perillyl alcohol have been shown to both prevent the development of carcinogenesis [18,22] in a variety of experimental rodent models and to cause regression [23, 24] of rat mammary carcinomas. *R*(+) limonene and *S*(−) perillyl alcohol are in clinical evaluation for the treatment of cancer patients [25].

In several mammalian species, including humans, limonene and perillyl alcohol are rapidly metabolized to perillic acid (PA) and dihydroperillic acid (major products) as well as to the methyl esters of the two acids (minor products) [23,26]. These metabolites display a greater pharmacological potency than limonene as inhibitors of protein prenyltransferases as well as of protein prenylation [14,16–18]. There is also a correlation between the ability of PA and perillyl alcohol to inhibit protein prenylation and proliferation of cancer cell lines [18]. These data raise the possibility that the *in vivo* antitumor effects of limonene and perillyl alcohol may be mediated by PA and by other metabolites through inhibition of protein prenylation.

In this study we report that exposure of SMC to concentrations of *S*(−) PA capable of decreasing protein prenylation leads to DNA synthesis inhibition, further supporting the role of prenylated proteins in the control of SMC proliferation.

2. Materials and methods

2.1. Materials

Eagle's minimum essential medium (MEM), trypsin ethylendiaminetetraacetate, penicillin (10,000 U mL^{-1}), streptomycin (10 mg mL^{-1}), tricine buffer (1 M, pH 7.4), and nonessential amino acid solution (100 ×) were purchased from Gibco (Life Technologies, Milano, Italy), and fetal calf serum (FCS) was from Mascia Brunelli (Milano, Italy). Disposable culture flasks and petri dishes were from Corning Glassworks, and filters were from Millipore. [2–^{14}C]acetate, sodium salt (58.9 mCi $mmol^{-1}$), [6–^{3}H]thymidine, sodium salt (2 Ci $mmol^{-1}$), and molecular weight protein standards were from Amersham. Isoton II was purchased from Instrumentation Laboratories. All-*trans* FOH, propidium iodide, and Hoechst 33342 were from Sigma, and all-*trans* GGOH was kindly provided by Prodotti Roche. sodium dodecyl sulfate (SDS), NNNN-tetra-methyl-ethylendiamine, ammonium persulfate, glycine, and acrylamide solution (30% T, 2.6%) were obtained from Bio-Rad Laboratories. All-*trans* FOH [1–^{3}H] (15–20 Ci $mmol^{-1}$) and all-*trans* GGOH [1–3H] [50–60 Ci $mmol^{-1}$) were from American Radiolabeled Chemicals. *S*(−) PA was synthesized by oxidation (CrO_3, AcOH, H_2O) of *S*(−) perillyl alcohol [28]. The *cis* and *trans* PAs were separated by chromatography, and their stereochemistry was established by high field ^{1}H nuclear magnetic resonance. *S*(−) PA was dissolved in dimethyl sulfoxyde (0.5% final concentration), and control cells received the same volume of the solvent. Simvastatin in its lactone form (Merck, Sharp, & Dohme Research Laboratories) was dissolved in 0.1 M NaOH to give the active form, and the pH was adjusted to 7.4 by adding 0.1 M HCl. The solution was sterilized by filtration.

2.2. Cell proliferation and DNA synthesis

SMC were cultured from the intimal-medial layers of aorta of male Sprague-Dawley rats as previously described [27].

Cell proliferation was evaluated by cell counting with a Coulter Counter model ZM (Coulter Instruments) after trypsinization of the monolayers [4], and DNA synthesis was estimated by nuclear incorporation of [^{3}H]thymidine [27].

2.3. Cell cycle analysis

Flow cytometry was used to analyze cell cycle distribution. Cells were trypsinized and centrifuged for 5 min at 1000 rpm. Pellets were resuspended in 0.5 mL hypotonic fluorochrome solution of propidium iodide (PI) 50 μg/mL in 0.1% sodium citrate containing 0.1% TritonX-100. Samples were placed in the dark for 30 min, and the PI fluorescence of individual nuclei was measured. Nuclear PI fluorescence signal was recorded on the FL2 channel of a

fluorescent activated cell sorter scan flow cytometer (Becton Dickinson), and DNA histogram analysis performed by using a Lysis II program. The number of cells in sub-G_1 (DNA content<2N), G_0/G_1, S, and G_2/M phases was expressed as percentages of total events (10,000 cells).

2.4. Apoptosis analysis

Sparse cells growing on glass slides were fixed for 5 min in cold methanol (−20°), rinsed twice in phosphate buffered saline (PBS), and subsequently incubated with Hoechst 33342 (5 μg/mL in PBS) for 30 min at room temperature in the dark [29]. Cells were photographed by using a Polyvar fluorescent microscope and DNA chromatin morphology under UV visualization. The percentage of cells showing nuclear features of apoptosis by Hoechst staining (chromatin condensation, fragmentation, or shrinkage) was evaluated in approximately 1000 nuclei for each population by using a Quantimet 920 image analyzer (Cambridge Instruments) connected to a Polyvar microscope by a Hamamatsu HC3077 camera and repeated in three different experiences [30].

2.5. Synthesis of total sterols

The synthesis of cholesterol was determined by measuring the incorporation of radioactive acetate into cellular sterols [27].

2.6. Labeling of proteins with [^{3}H]FOH or [^{3}H]GGOH and SDS-PAGE analysis

The prenylated proteins were analyzed after incubation of the cell monolayers with [^{3}H]FOH (10 μM) or [^{3}H]GGOH (2.5 μM) for 5 h (see: Experimental protocols) according to Corsini *et al.* [7]. After this time, cell monolayers were scraped into 1.5 mL of PBS containing 1 mM phenylmethylsulphonylfluoride, and the resuspended cells collected and centrifuged (14000 rpm × 3 min). Cells pellets were delipidated with cold acetone, followed by two different extractions with chloroform/methanol (2:1). The delipidated proteins were solubilized in 3% SDS, 62.5 mM Tris-HCl, pH 6.8, and an aliquot (40 μg protein) was analyzed by one-dimensional 12.5% SDS-polyacrylamide gel electrophoresis (PAGE), according to Corsini *et al.* [7]. After electrophoresis, the gel was treated with Amplify (Amersham) and exposed to Kodak X-Omat-AR film at −70° for 2–4 weeks. Fluorographic signals were analyzed by densitometric scanning.

2.7. Experimental protocols

2.7.1. Proliferation, cell cycle analysis, and cholesterol synthesis in SMC

Cells were seeded at various density (1–3 x 10^5) SMCs/petri dish (35 mm) and incubated with MEM supplemented with 10% FCS. Twenty-four h later, the medium was changed to one containing 0.4% FCS to stop cell growth, and the cultures were incubated for 72 h. At this time (time 0), the medium was replaced by one containing 10% FCS, in the presence or absence of known concentrations of the tested compound, and the incubation was continued for further 24–72 h, at 37°. At time 0, just before the addition of the substances to be tested, three petri dishes were used for cell counting. SMC proliferation was evaluated by cell counting, with a Coulter Counter model ZM, after trypsinization of the monolayers [4], and SMC doubling time was computed. Under the same experimental conditions, cell cycle analysis and nuclear chromatin morphology were evaluated after incubation for 72 h with the tested compound. In a subsequent set of experiments, synchronization of SMC to the G_0/G_1 interphase of the cell cycle was accomplished by incubating logarithmically growing cultures (3×10^5 cells/plate) for 120 h in a medium containing 0.4% FCS. Quiescent cells were incubated for 20 h in a fresh medium with 10% FCS, in the presence of the tested compound. DNA synthesis was then estimated by nuclear incorporation of [^{3}H]thymidine, incubated with cells (2 μCi mL^{-1}) for 2 h, as previously described [27]. Under the same experimental conditions, in another set of petri dishes, cholesterol biosynthesis was estimated by measuring the incorporation of [^{14}C]acetate into cellular sterols [27]. Cell viability was assessed by trypan blue exclusion and found to be higher than 95% at the concentrations of *S*(−) PA used.

2.7.2. Cell labeling and prenylated proteins analysis

Cells were incubated for 20 h under the same experimental conditions used for investigating [^{3}H]thymidine incorporation in the presence or absence of the tested compound. [^{3}H]FOH or [^{3}H]GGOH were added during the last 5 h of incubation. Cell pellets were delipidated, and equal amounts of cell extracts (40 μg cell protein/lane) were separated by 12.5% SDS-PAGE and fluorographed [7].

2.7.3. Statistical analysis

Experimental data are expressed as mean ± S.D. The effects of the tested compounds versus control on the different parameters were analyzed by two-tailed Student's test for unpaired data, by a two-way ANOVA followed by Dunnett multiple comparison test.

3. Results

3.1. Effect of S(−) PA on arterial SMC proliferation and apoptosis

The effect of *S*(−) PA (1- 3.5 mM), the metabolite of the naturally occurring monoterpene perillyl alcohol [16], on the proliferation of rat SMC as related to protein prenylation was investigated. In a first set of experiments, the ability of the tested compound was evaluated by cell counting after

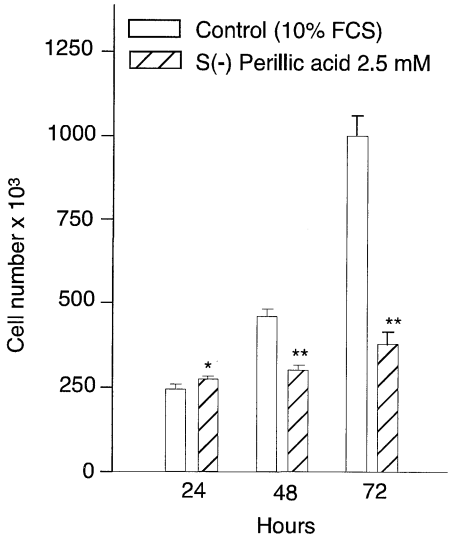

Fig. 1. Time-dependent effect of *S*(−) PA on proliferation of rat aorta smooth muscle cells. Cells were seeded at a density of 100×10^3/dish and incubated with Eagle's MEM supplemented with 10% FCS; 24 h later the medium was changed to one containing 0.4% FCS to stop cell growth, and the cultures were incubated for 48 h. At this time (time 0, cell number 184×10^3), the medium was replaced with one containing 10% FCS in the presence or absence of *S*(−)PA 2.5 mM. The incubation was continued for 24, 48, and 72 h, respectively. Each bar represents the mean ± S.D. of triplicate dishes. Inhibitor versus control: * $P < 0.05$; ** $P < 0.01$; (Dunnett test).

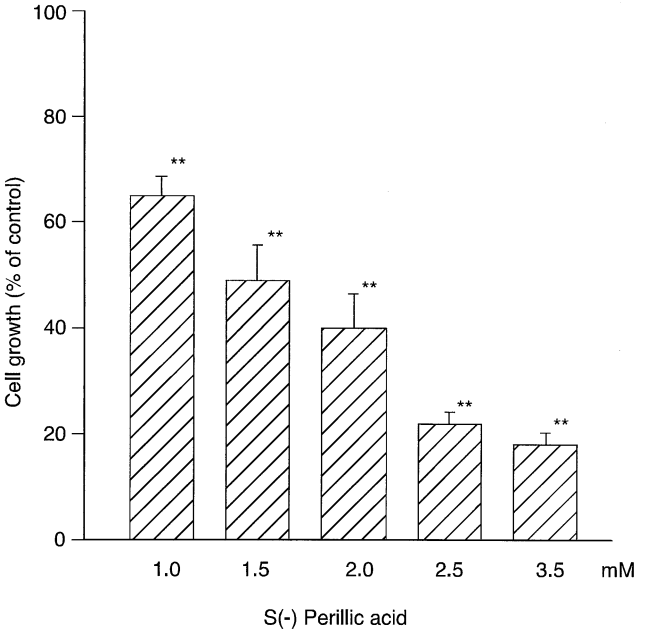

Fig. 2. Concentration-dependent effect of *S*(−) PA on proliferation of rat aorta smooth muscle cells Cells were seeded at a density of 200×10^3 /dish and incubated with Eagle's MEM supplemented with 10% FCS; 24 h later the medium was changed to one containing 0.4% FCS to stop cell growth, and the cultures were incubated for 48 h. At this time (time 0, cell number 272×10^3), the medium was replaced with one containing 10% FCS and the reported concentrations of the compound and the incubation was continued for further 72 h at 37°. Each bar represents the mean ± S.D. of triplicate dishes. The mean values for control experiments (without inhibitor) was 1467×10^3 ($\pm 89 \times 10^3$) cells/dish. Inhibitor versus control: ** $P < 0.01$ (Dunnett test).

several days of exposure to the drug. *S*(−) PA inhibited the growth of arterial SMCs in a time-dependent manner, reaching 65% inhibition after 3 days in the presence of 2.5 mM of *S*(−) PA (Fig. 1). Treated cells had a higher doubling time than did control cells**,** except for *S*(−) PA 2.5 mM at time point 24 h (Table 1). *S*(−) PA decreased rat SMC proliferation in a concentration-dependent manner after 72 h of drug exposure (Fig. 2). As shown in Fig. 3, *S*(−) PA also inhibited the nuclear incorporation of thymidine by rat SMC in a concentration-dependent manner. Flow cytometry analysis of cell cycle showed that *S*(−) PA 2.5 mM induced the block of the cell cycle progression in G_0/G_1 interphase (Fig. 4). The percentage of *S*(−) PA-treated SMCs in G_0/G_1 (87.1) was similar to that observed in quiescent cultured cells (79.6), while in the presence of 10% FCS a progression to S and G_2/M phases was detected (Fig. 4). Moreover, the percentage of sub G_0/G_1 cells treated with 2.5 mM *S*(−) PA was low and similar to that of control cells (0.7 vs. 0.5%, respectively; Fig. 4). In the presence of 0.4% FCS, this percentage increased (6.1%, $P < 0.01$, Student's *t* test). To verify the presence of cells with morphological features of apoptosis (Fig. 4) we calculated the percentage of condensed or fragmented nuclei in sparse adherent cultures stained with Hoechst 33342. After 72 h of treatment with 2.5 mM of *S*(−) PA, the percentage of apoptotic SMCs was 0.9 ± 0.3, similar to that of control SMCs (0.8 ± 0.25). In the presence of 0.4% FCS alone, the percentage of apoptotic cells was increased (5.2 ± 1.6, $P < 0.01$, Student's *t* test).

Table 1
Mean doubling time for SMC cultured from rat aorta: effect of S(−) PA

Treatment	Doubling time (h ± S.D.)
24 h	
Control	58.27 ± 3.8
S(−) PA 2.5 mM	41.62 ± 1.4**
S(−) PA 3.5 mM	122.85 ± 7.3***
48 h	
Control	36.26 ± 2.1
S(−) PA 2.5 mM	68.26 ± 4***
S(−) PA 3.5 mM	130.7 ± 10***
72 h	
Control	29.4 ± 1.6
S(−) PA 2.5 mM	69.57 ± 7.3***
S(−) PA 3.5 mM	269.8 ± 23.1***
96 h	
Control	32.48 ± 4.35
S(−) PA 2.5 mM	85.65 ± 2.5***
S(−) PA 3.5 mM	215.83 ± 10.7***

** $P < 0.01$; *** $P < 0.001$ (Student's test).

3.2. Effect of S(−) PA on the MVA pathway

To better define the mechanism of *S*(−) PA's antiproliferative activity, we investigated the effect of this compound

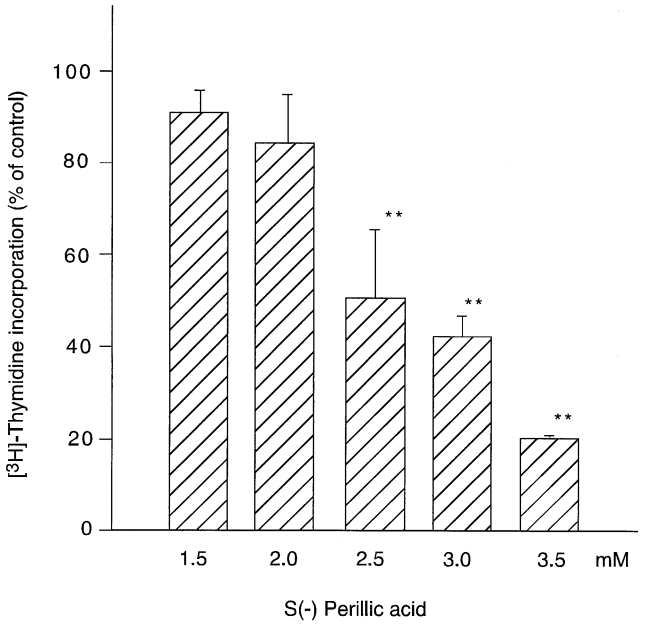

Fig. 3. Concentration-dependent effect of *S*(−) PA on thymidine incorporation by rat aorta smooth muscle cells. Cells were seeded at a density of 300×10^3/dish and incubated with Eagle's MEM supplemented with 10% FCS; 24 h later, the medium was changed with one containing 0.4% FCS to stop cell growth, and the cultures were incubated for 120 h. At this time, the medium was replaced with one containing 10% FCS and the reported concentrations of *S*(−) PA. After 20 h, at 37°, labeled thymidine was added to the medium and the incubation continued for further 2 h. Each bar represents the mean ± S.D. of triplicate dishes. The mean value for control experiments (without inhibitor) was 494×10^3 ($\pm$ 47×10^3) dpm mg prot^{-1}. Inhibitor versus control: ** $P < 0.01$ (Dunnett test).

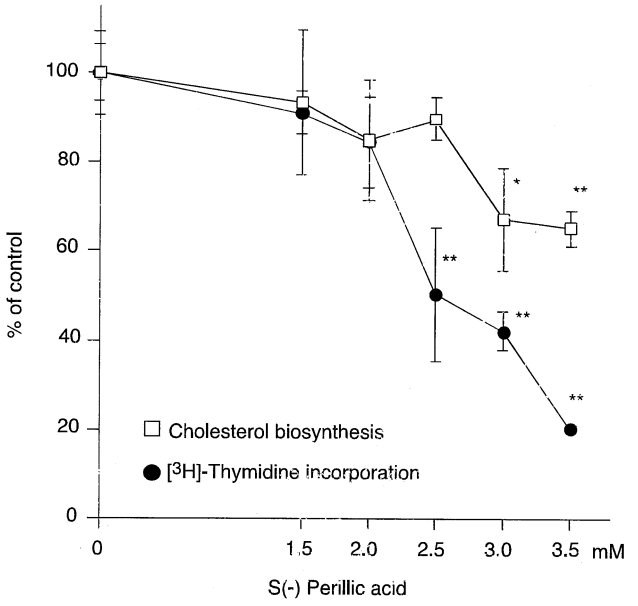

Fig. 5. Effect of *S*(−) PA on thymidine incorporation and cholesterol biosynthesis by rat aorta smooth muscle cells. Experimental conditions are as in Fig. 3. ^{14}C-acetate incorporation was used to assay cholesterol biosynthesis, 20 h after its addition to the cells. Each point represents the mean ± S.D. of triplicate dishes. The mean value for control experiments (without inhibitor) for thymidine incorporation was 494×10^3 ($\pm$ 47×10^3) dpm mg prot^{-1} and 43.41 ($\pm$ 2.77) pmol mg^{-1} × h for cholesterol biosynthesis, respectively. Inhibitor versus control: * $P < 0.05$; ** $P < 0.01$ (Dunnett test).

on cholesterol biosynthesis, a major end-product of the MVA pathway. As shown in Fig. 5, *S*(−) PA inhibited DNA synthesis but caused only a slight, although significant, decrease in cholesterol biosynthesis in SMC.

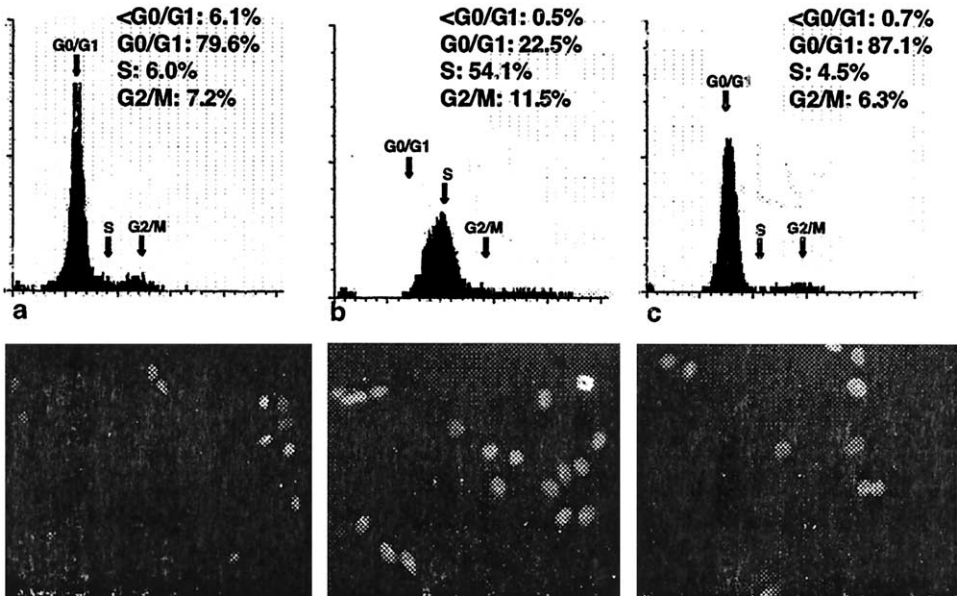

Fig. 4. Effect of S(−) PA on cell cycle and apoptosis of rat aorta smooth muscle cells. Experimental conditions are as in Fig. 2. Flow cytometry analysis of cell cycle was performed in the presence of (a) 0.4% and (b) 10% FCS alone or in the presence of *S*(−) PA 2.5 mM. DNA content and morphology of SMCs after Hoechst 33342 staining in the presence of (d) 0.4% and (e) 10% FCS alone or (f) in the presence of *S*(−) PA 2.5 mM. Arrow indicates a mitotic figure (fluorescent microscopy, 160 ×).

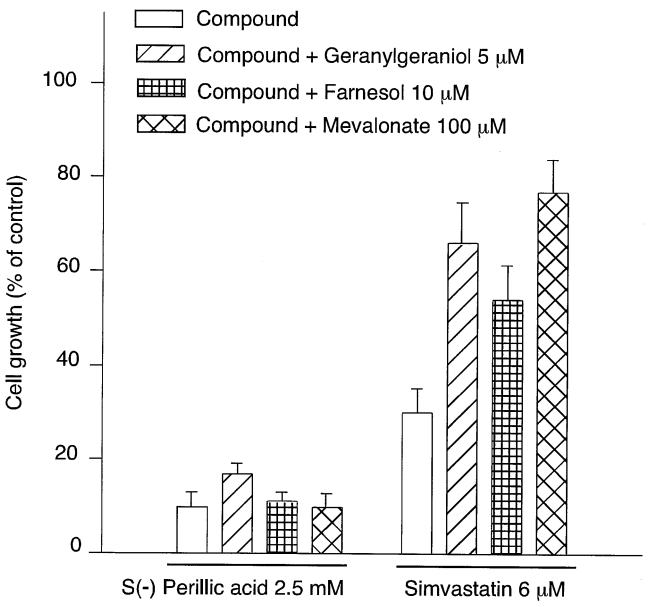

Fig. 6. Effect of MVA and its derivatives on proliferation of rat aorta smooth muscle cell inhibited with *S*(−) PA or simvastatin. Experimental conditions are as in Fig. 2. Each bar represents the mean ± S.D. of triplicate dishes. The mean value for control experiments (without inhibitor) was 2040×10^3 ($\pm$ 123×10^3) cells/dish.

We then investigated the ability of MVA, FOH, and GGOH to counteract the inhibitory effect of *S*(−) PA on SMC proliferation. As shown in Fig. 6, the antiproliferative effect of *S*(−) PA, in contrast to that observed with simvastatin [5], was not prevented by the addition of MVA, FOH, and GGOH. These differences between simvastatin and *S*(−) PA strongly suggest that the antiproliferative effect of *S*(−) PA is not due to inhibition of HMG-CoA reductase.

To investigate whether PFTase and PGGTase might be the targets for *S*(−) PA's action, the incorporation of [^{3}H]FOH and [^{3}H]GGOH into cellular proteins [7] was examined under the same experimental conditions used for evaluating DNA synthesis. As shown in Figs. 7 and 8, [^{3}H]FOH and [^{3}H]GGOH were readily incorporated into specific proteins with molecular weights ranging from 21 to 72 kDa, as also reported in previous studies [6,7]. *S*(−) PA inhibited [^{3}H]FOH incorporation into specific low molecular weight proteins in a concentration-dependent manner (Fig. 7). In each experiment, simvastatin was used to block endogenous MVA synthesis, to increase the specific radioactivity of the [^{3}H]FOH derived [^{3}H] Farnesyl Pyrophosphate (FPP) pool for efficient radiolabeling of proteins [7]. *S*(−) PA was also able to inhibit the incorporation of [^{3}H]GGOH into specific proteins in a concentration-dependent manner (Fig. 8). Similar results were obtained when the effect of *S*(−) PA (3.5 mM) on protein farnesylation (−69% ± 11 vs. control, $P < 0.01$, Student's *t* test) and geranylgeranylation (−74% ± 7 vs. control, $P < 0.01$, Student's *t* test) was investigated in two other separate experiments (data not shown). In agreement with previous findings in other cell lines [18], similar concentrations of *S*(−) PA were required to inhibit both DNA synthesis and protein prenylation. Taken together, these results support that inhibition of protein prenylation is, at least, one of the mechanisms underlying the antiproliferative activity of *S*(−) PA.

4. Discussion

In the present study, the effect of *S*(−) PA on arterial myocyte proliferation as related to protein prenylation was

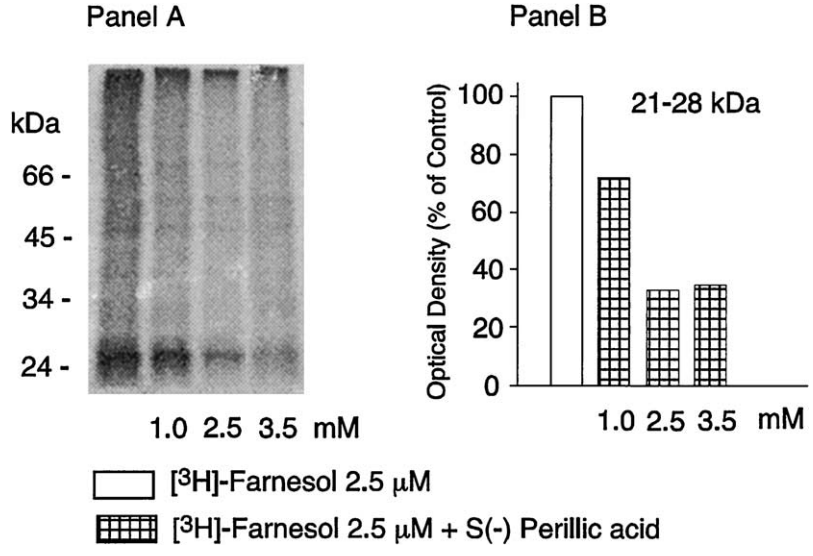

Fig. 7. Concentration-dependent effect of *S*(−) PA on [^{3}H]-FOH incorporation into proteins of rat aorta smooth muscle cells. Experimental conditions are as in Fig. 3. Quiescent cells were incubated for 20 h in a fresh medium containing 10% FCS and 5 μM simvastatin in the presence or absence of *S*(−) PA. [^{3}H]FOH (10 μM) was added during the last 5 h of incubation. Cell pellets were delipidated, and equal amounts of cell extracts (40 μg cell protein/lane) were separated by 12.5% SDS-PAGE and fluorographed (Panel A). The resulting film was analyzed by densitometry (Panel B).

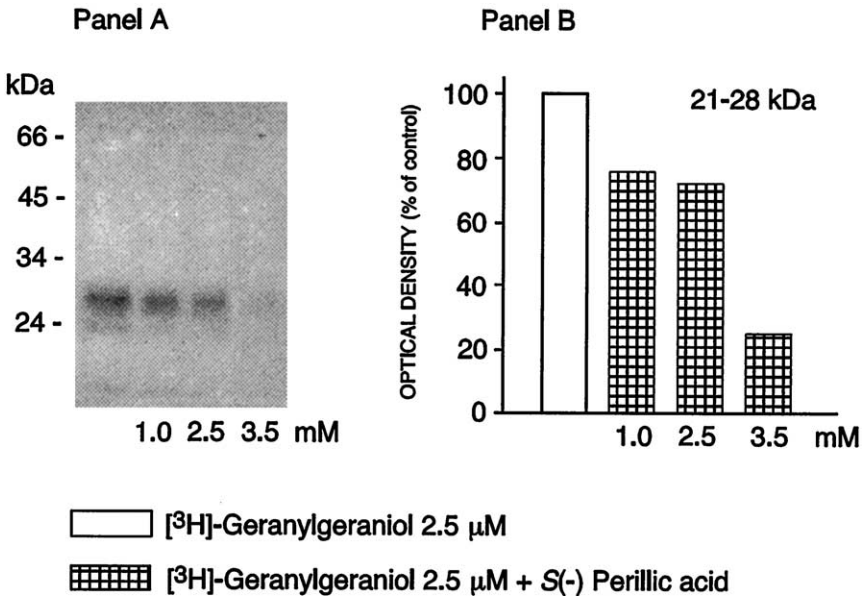

Fig. 8. Concentration-dependent effect of *S*(−) PA on [^{3}H]-GGOH incorporation into proteins of rat aorta smooth muscle cells. Experimental conditions as in Fig. 3. Quiescent cells were incubated for 20 h in a fresh medium containing 10% FCS in the presence or absence of *S*(−) PA. [^{3}H]GGOH (2.5 μM) was added during the last 5 h of incubation. Cell pellets were delipidated, and equal amounts of cell extracts (40 μg cell protein/lane) were separated by 12.5% SDS-PAGE and fluorographed (Panel A). The resulting film was analyzed by densitometry (Panel B).

evaluated. The results indicate that *S*(−) PA is effective at reducing the proliferation of rat arterial myocytes. According to previous studies [19,31], both flow cytometry analysis and DNA synthesis assay showed that *S*(−) PA specifically blocks the cell cycle in G_1, reducing the percentage of cells in S and G_2/M phase. A similar inhibitory effect on the G_1 phase has been observed in cells treated with HMG-CoA reductase inhibitors [32], with PFTase inhibitors [33], and with PGGTase I inhibitors [34]. These results suggest the involvement of specific isoprenoid metabolites, probably through farnesylated and geranylgeranylated proteins, in the G_1/S phase transition of the cell cycle. The absence of hypodiploid cells and condensed or fragmented nuclei exclude the possibility that apoptosis could contribute to the action of *S*(−) PA. However, the above referred classes of compounds i.e. statins [35] and terpenes [36], including only a high concentration (10 mM) of *S*(−) PA [35], as well as PFTase inhibitors and PGGTase inhibitors [34] can induce apoptosis in cultured cells. The discrepancy between our results and the above studies could be explained by the different concentration of the tested compound (2.5 vs. 10 mM) and by the different experimental conditions used.

The present results extend our knowledge regarding the effect of *S*(−) PA on cell proliferation for, at least, two reasons: first, this report demonstrates the ability of *S*(−) PA to inhibit the growth of untransformed primary cells, specifically vascular SMC. Most previous studies with *S*(−) PA were carried out with a variety of tumor cell lines. The exception is one recent demonstration that mM concentrations of *S*(−) PA cause inhibition of primary phytohemagglutinin-stimulated human lymphocytes [19]. In all of these studies, *S*(−) PA exhibits a similar potency (sub to low millimolar range), thus suggesting a nonselective antimitotic effect. Interestingly PGGTase I inhibitors have been reported to decrease SMC proliferation [34]. On the other hand, the effect of PFTase inhibitors on SMC proliferation is disputable. Although Unlu [37] and Stark [34] failed to show any inhibitory effect, Raiteri [5] demonstrated a significant reduction of SMC proliferation caused by PFTase inhibitors. The latter observation is consistent with an induction of farnesylated H-ras protoncogene expression in proliferating rat SMC [38] and with the role of prenylated proteins in the transduction of mitogenic stimuli through G-protein coupled receptors in SMC [39].

Second, the ability of *S*(−) PA to significantly inhibit SMC proliferation occurs at concentrations (1–3 mM) similar to those observed in plasma (0.47 mM) of patients treated with perillyl alcohol [25]. Moreover, in earlier investigation we were able to also detect a nonsignificant inhibition of SMC growth (−7 and −15%) by using 0.1 and 0.5 mM *S*(−) PA, respectively (data not shown). This effect could provide a basis for a potential interest of the compound in reducing SMC proliferation, a key event occurring after coronary angioplasty and stent implantation [40]. It is important to mention that perillyl alcohol is rapidly metabolized to *S*(−) PA, which is one of its main metabolites [25]. The mechanism whereby *S*(−) PA and other monoterpenes affect cell proliferation is still under study, but several drug-related activities have been documented. Monoterpenes have been reported to interfere with several enzymatic steps of the MVA pathway, e.g. MVA synthesis by HMG-CoA reductase [41], conversion of lathosterol to cholesterol [42], synthesis of coenzyme Q [42], and protein prenylation reaction via protein prenyltransferases [14]. A

major result of the current study is the demonstration that *S*(−) PA significantly inhibits DNA synthesis at concentrations that only slightly reduce cholesterol biosynthesis. The relevance of this mild inhibitory effect still remains to be explored. Another approach for understanding the relationship between the MVA pathway and the antiproliferative effect of *S*(−) PA makes use of MVA and its isoprenoid derivatives. We previously showed [4,5] that the addition of MVA, all-*trans* FOH, and all-*trans* GGOH, precursors of prenylated proteins [3], restored cell proliferation inhibited by statins, supporting a specific role of isoprenoids in regulating cell proliferation. In contrast to results observed with statins, the addition of MVA, all-*trans* FOH, and all-*trans* GGOH did not counteract *S*(−) PA-induced blockade of SMC proliferation; this lends further support to the hypothesis that PFTase and PGGTases are very likely targets for *S*(−) PA's action. To directly address the effect of *S*(−) PA on protein prenylation, we examined the incorporation of labeled all-*trans* FOH and all-*trans* GGOH into cell proteins. Different studies [6,7,13] have clearly shown that [^{3}H]FOH and [^{3}H]GGOH are readily incorporated into specific cellular proteins and into other metabolites of the MVA pathway made from prenyl-pyrophosphate (PP), thus supporting our experimental approach. When proteins are labeled with [^{3}H]FOH or [^{3}H]GGOH and subsequently analyzed by one-dimensional SDS-PAGE, several major bands of prenylated proteins can be distinguished: a subset of proteins with molecular weights ranging from 21 to 72 kDa, that incorporates [^{3}H]FOH, and a subset of proteins with a molecular weight of 21–28 kDa that incorporates [^{3}H]GGOH [7]. *S*(−) PA has been shown to inhibit the incorporation of both labeled isoprenols, [^{3}H]FOH and [^{3}H]GGOH, into high and low molecular weight proteins. This observation leads us to conclude that, in our experimental conditions, the inhibition of protein prenylation by *S*(−) PA is neither specific nor selective for a particular class of proteins. These results are in contrast to previous observations [17] showing that monoterpenes inhibit protein prenylation of a class of cellular proteins of 21–28 kDa, including p21ras and possibly other small Guanosine 5-Triphosphate (GTP)-binding proteins, without affecting high molecular weight prenylated proteins. The discrepancy between our results and the above study could be related to the use of labeled isoprenols, instead of [^{3}H]MVA, and to the different cell lines investigated. However, other authors [19] have already shown that cells treated with PA exhibit a decrease in the incorporation of [^{3}H]MVA into proteins in PA-treated cells. The mechanism by which terpenes, including PA, interfere with the incorporation of MVA and isoprenoids into specific proteins is potentially related to the inhibition of protein prenylation enzymes PFTase and PGGTases I and II [15,16]. Therefore, *S*(−) PA is expected to block the binding of FPP and GGPP to their respective protein prenyltransferases and thus should inhibit the prenylation of most, if not all, proteins modified by these enzymes. Nevertheless, it is important to mention that *S*(−) PA (5 mM) possesses greater activity toward PGGTase I (50% inhibition) than toward PFTase (20% inhibition) [14,16]. The observation that *S*(−) PA blocks SMC proliferation at concentrations that inhibit protein prenylation supports the involvement of specific prenylated proteins in PA-induced inhibition of cell growth. It remains to be determined which particular protein(s) are involved in the antiproliferative activity of *S*(−) PA in this *in vitro* model. This matter needs to be further examined by means of two-dimensional electrophoresis as well as western blotting analysis of specific proteins candidates. It is noteworthy to mention that other mechanism(s) of the antiproliferative action of *S*(−) PA cannot be ruled out. For example, molecular studies have reported an increase in mannose 6-P/insulin growth factor II receptor expression, associated with an increase in TGF-β expression, leading to the antitumor activity of these compounds [43,44], as well as to the induction of apoptosis [35].

In summary, our data indicate that *S*(−) PA is able to inhibit SMC proliferation in a time- and concentration-dependent manner. Altogether, we provide evidence that *S*(−) PA affects protein prenylation, an effect that may contribute to its inhibition of SMC proliferation.

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