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ORIGINAL ARTICLE

Polyamine synthesis inhibition induces S phase cell cycle arrest in vascular smooth muscle cells

M. Odenlund · B. Holmqvist · B. Baldetorp · P. Hellstrand · Bengt-Olof Nilsson

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Abstract Polyamines are important for cell growth and proliferation and they are formed from arginine and ornithine via arginase and ornithine decarboxylase (ODC). Arginine may alternatively be metabolised to NO via NO synthase. Here we study if vascular smooth muscle cell proliferation can be reversed by polyamine synthesis inhibitors and investigate their mechanism of action. Cell proliferation was assessed in cultured vascular smooth muscle A7r5 cells and in endothelium-denuded rat arterial rings by measuring [^{3}H]-thymidine incorporation and by cell counting. Cell cycle phase distribution was determined by flow cytometry and polyamines by HPLC. Protein expression was determined by Western blotting. The ODC inhibitor DFMO (1–10 mM) reduced polyamine concentration and attenuated proliferation in A7r5 cells and rat tail artery. DFMO accumulated cells in S phase of the cell cycle and reduced cyclin A expression. DFMO had no effect on cell viability and apoptosis as assessed by fluorescence microscopy. Polyamine concentration and cellular proliferation were not affected by the arginase inhibitor NOHA (100–200 μM) and the NO synthase inhibitor L-NAME (100 μM). Lack of effect of NOHA was reflected by absence of arginase expression. Polyamine synthesis inhibition attenuates vascular smooth muscle cell proliferation by reducing DNA synthesis and accumulation of cells in S phase, and may be a useful approach to prevent vascular smooth muscle cell proliferation in cardiovascular diseases.

Keywords Arginase · Cell cycle distribution · DNA synthesis · Ornitihine decarboxylase · Polyamines · Vascular smooth muscle cells

M. Odenlund · P. Hellstrand · B.-O. Nilsson (✉)
Department of Experimental Medical Science,
Division of Vascular and Airway Research,
Unit of Vascular Physiology, Lund University,
BMC D12, 221 84 Lund, Sweden
e-mail: bengt-olof.nilsson@med.lu.se

B. Holmqvist · B. Baldetorp
Department of Clinical Sciences, Unit of Oncology,
Lund University Hospital, Lund University,
221 85 Lund, Sweden

Introduction

In atherosclerosis inflammatory mediators, cytokines and growth factors profoundly affect vascular smooth muscle and endothelial cell functional properties (Libby 2002; Ross 1993). A key event in the pathogenesis is migration and proliferation of vascular smooth muscle cells from the media into the intima. Polyamines are found in all living cells and are essential for cellular proliferation (Pegg and McCann 1982). They are formed from ornithine in a sequence of enzymatic reactions. The rate-limiting step in polyamine biosynthesis is the decarboxylation of ornithine via ornithine decarboxylase (ODC) into putrescine (PUT). PUT is enzymatically converted to the higher polyamines spermidine (SPD) and spermine (SPN) via addition of first one and then another aminopropyl group with decarboxylated S-adenosylmethionine serving as the aminopropyl group donor (Pegg and McCann 1982). Inhibition of ODC with α-difluoromethylornithine (DFMO) has been reported to reduce the cellular level of polyamines and to arrest cellular proliferation in cultured cancer cells (Pegg 1986; Pegg et al. 1995a). Undoubtedly, polyamines are necessary for cellular proliferation and growth, but their mechanism of action is not clear.

Besides taking up ornithine from the extra cellular space, cells may produce ornithine from arginine. Arginase, which exists in two isoforms, arginase I and II, catalyses the conversion of arginine to ornithine. Increased rat aortic vascular smooth muscle cell expression of arginase I by transfection stimulates cell proliferation by a mechanism involving increased cellular concentration of polyamines (Wei et al. 2000). The cytokines IL-4 and IL-13 induce arginase I expression, enhance polyamine formation and stimulate vascular smooth muscle cell proliferation (Wei et al. 2000; 2001). These studies suggest a correlation between inflammation, elevated arginase I expression, polyamine formation, and enhanced proliferation in vascular smooth muscle cells. Arginine acts as substrate not only for arginase and polyamine formation but also for the NO synthases (eNOS, iNOS and nNOS) catalysing the formation of NO. The principal intermediate in this pathway N^G-hydroxy-L-arginine (NOHA) is a competitive inhibitor of arginase (Buga et al. 1996; Daghigh et al. 1994).

Polyamines have also been reported to be implicated in other cellular functions than cell growth and proliferation, such as regulation of ion channel properties (Ahern et al. 2006; Nilsson et al. 2002; Williams 1997), apoptosis (Wallace et al. 2003) and cell migration (Liang et al. 2004; Ray et al. 2003; Wang et al. 2000; Yuan et al. 2000). They have been shown to be crucial for intestinal epithelial cell migration (Ray et al. 2003). We have shown that treatment with polyamine synthesis blockers reduces mouse aortic smooth muscle cell migration but their mechanism of action is unknown (Liang et al. 2004). Interestingly, a recent study by Dumont et al. (2007) shows that ODC antizyme 1 gene polymorphism is associated with sub-clinical and clinical vascular events implicating that enhanced polyamine formation is involved in the pathogenesis of vascular disease.

The objective of the present study was to investigate if interference with arginine and polyamine metabolism using enzyme inhibitors affects vascular smooth muscle cell proliferation and to investigate their mechanism of action. We report that treatment with the ODC inhibitor DFMO causes polyamine depletion, attenuates DNA synthesis and accumulates vascular smooth muscle cells in the S phase of the cell cycle probably via attenuation of cyclin A expression. DFMO reduces not only proliferation of cultured vascular smooth muscle cells but also PDGF-AB-induced cell proliferation in endothelium-denuded arterial rings. These data suggest that pharmacological intervention targeting polyamine biosynthesis is a possible way to prevent unwanted vascular smooth muscle cell proliferation.

Materials and methods

Cells and tissue

The vascular smooth muscle cells (A7r5 cell line, ATCC, Manassas, VA, USA) derived from embryonic rat aorta were grown in a mixture (1:1) of Dulbeccos Modified Eagle's Medium and Ham's F12 medium with addition of antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin) and foetal calf serum (FCS, 10%) in a water-jacketed cell incubator under 5% CO_2 in air. The A7r5 cells were subcultivated after reaching confluence and used in passages 3–5. Before start of experiments, at a cell confluence of about 50%, the normal culture medium was replaced with low arginine-containing (21 mg/ml in low-arginine vs. 148 mg/ml in normal culture medium) culture medium. The cells were made quiescent at 60–80% confluence by removing FCS for 24 h. They were then pre-incubated for 2 h in FCS-free medium with or without the arginase inhibitor N^G-hydroxy-L-arginine (NOHA), the ODC inhibitor α-difluoromethylornithine (DFMO) and the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) before 5% FCS was introduced. The cells were treated for 24 h up to 5 days with or without the different drugs. For fluorescence microscopy the cells were allowed to grow on glass cover slips.

Tail artery and aorta were removed from 14 female Spraque–Dawley rats, of 2 months' age and weighing 200 g. The experiments were approved by the Animal Ethics Committee at Lund University. The endothelium was removed by moving a fine needle up and down the vessel lumen. The tail arteries were cut into rings (2–3 mm wide) and placed in culture dishes with culture medium as described above. The same experimental conditions were applied in experiments on A7r5 cells and rat tail arterial rings except that PDGF-AB (100 ng/ml) instead of FCS (5%) was used as proliferation promoter in arterial rings as described by Lindqvist et al. (2001). Tissue culture of vascular preparations to preserve the vascular wall cells in their natural environment has been used extensively in our laboratory (Lindqvist et al. 1997, 2001; Zeidan et al. 2000) and by others (Chamley-Campbell et al. 1981; De Mey et al. 1989; Schiotz et al. 2000) to study vascular contractility, growth and differentiation.

Protein extraction and Western blotting

The cells were scraped off the culture dish, washed in PBS, centrifuged (4,000 rpm for 5 min at 4°C), and then suspended in sample buffer (Tris–HCl 62.5 mM, pH 6.8, 2% SDS, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride). Rat tail artery and aorta were cleaned, rinsed, de-endothelialized, snap-frozen in liquid nitrogen and then

suspended in sample buffer. After sonication (2 × 10 s) the samples were boiled and centrifuged at 14,000 rpm at 4°C for 10 min. Supernatants were collected and their protein concentrations determined using a Bio-Rad protein determination kit based on the Lowry method (Lowry et al. 1951). Proteins were separated by SDS-PAGE using 10 or 12.5% ready-gels (Bio-Rad, Hercules, CA, USA). In each lane the same amount of protein was loaded allowing comparisons of individual protein bands on different lanes. For loading control the gels were silver-stained after electrophoresis and analysed by photodensitometric scanning using a CCD camera (Bio-Rad Fluor-S Multiimager). After separation, proteins were transferred to a nitrocellulose membrane by electroblotting overnight at 4°C. The membrane was blocked for 1 h in 5% non-fat dry milk and then incubated overnight at 4°C with mouse monoclonal eNOS or arginase I antibodies (Transduction Laboratories, Lexington, KY, USA) at dilution 1:500 and 1:1,000, respectively, or with a rabbit polyclonal anti cyclin A antibody (Santa Cruz Biotechnology, Santa Cruz, USA) at dilution 1:1,000. The site of immunoreaction was visualized by chemiluminescence using HRP-conjugated secondary anti-mouse or anti-rabbit antibodies and chemiluminescence reagent (Cell Signaling Technology, Beverly, MA, USA). The immunospecific bands were analysed by photodensitometric scanning. Endothelial cell lysate and rat liver was used for positive control for eNOS and arginase I, respectively. For cyclin A human breast cancer SK-BR-3 cell lysate was used as positive control. For negative controls the respective primary antibody was omitted. For each protein every experiment was repeated at least twice.

Determination of polyamine and protein concentrations, cell number and cell viability

Polyamines were determined in cellular homogenate by HPLC as described by Seiler and Knodgen (1980). Cellular polyamine concentration was expressed as nmol/mg total protein. Number of cells was determined by cell counting in a Bürker chamber after trypsination (0.25%). Cell viability was determined by the trypan blue exclusion test. Cells were washed in 0.9% NaCl and incubated for 2 min with 0.4% trypan blue. The cells were then washed three times to remove unspecific staining and the number of stained cells was determined.

Determination of DNA synthesis

DNA synthesis was determined by measuring the incorporation of methyl-[^{3}H]thymidine (10 μCi, Amersham Biosciences, Uppsala, Sweden) into newly synthesized DNA as described (Jönsson et al. 2005; Lindqvist et al. 2001). The A7r5 cells and arterial rings were incubated with radiolabelled thymidine for 24 h. Radioactivity was expressed as c.p.m. and normalized to total protein concentration or tissue weight.

Imaging of cell morphology and cell viability

Immunofluorescence labelling techniques and fluorescence microscopy were used for determination of morphology and viability of cells treated with or without DFMO. After washing in PBS the glass, cover slips were immersed in 4% paraformaldehyde for 20 min at 4°C and rinsed. The cells were double-labelled with DAPI (DNA nuclear labelling) and phalloidin (F-actin labelling) conjugated with either Alexa 488 or Texas red (all from Invitrogen, Molecular Probes, Carlsbad, CA, USA).

For visual detection of apoptosis or other types of DNA fragmentation DNA strand breaks were determined using TUNEL staining kits (Texas red or fluorescein isothiocyanate, FITC, conjugated, Roche Molecular Biochemicals, Mannheim, Germany). TUNEL labelling was performed as single labelling for visualization of TUNEL signal only, or together with nuclear and cytoskeleton labelling for simultaneous visualization. TUNEL labelling specificity tests were made on separate slides in parallel by excluding the reaction mixture (negative control) and by treating cells with DNase I (positive control). The TUNEL labelling was compared to DAPI labelled cell nuclei and DNA fragments, and to the phalloidin-Texas red labelled F-actin cytoskeleton. Both single TUNEL labelling (including visualization by two different fluorophores, FITC or Texas red) and multiple labelling (TUNEL, DAPI and phalloidin) were performed in three separate experiments. Microscope analysis was performed with an Olympus microscope (AX60) for epi-fluorescence, using filter sets for blue emission (DAPI), green emission (Alexa 488) and red emission (Texas red). Digital images were grabbed separately from each channel and from overlays by the Olympus DP managar software.

Flow cytometry

After washing in PBS, cells were scraped off from the culture dish. The cells were then exposed to a cocktail containing hypotonic buffer with detergent, ribonuclease (Sigma-Aldrich, Stockholm, Sweden) and pepsin solution (Merck, Darmstadt, Germany). The nuclei were then stained with propidium iodide (Sigma-Aldrich, Stockholm, Sweden). Flow cytometric DNA analysis was performed in a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) equipped with the Cell Quest PRO™ program for the data acquisition. Excitation of propidium iodide was performed at

488 nm and fluorescence beyond 620 nm was detected. DNA contents were determined in about 13,000 nuclei in each sample. Processor signals were digitised and sorted into frequency distribution, DNA histograms, with a resolution of 256 units. Cell cycle phase distribution of the analysed cell population was determined by using ModFit LT 3.1, Verity Software House Inc. (Topsham, ME, USA) on the DNA histograms.

Drugs

DFMO was kindly provided by Hoechst Marion Roussel, Cincinnati, OH, USA. NOHA and L-NAME were from Sigma, St Louis, MO, USA. PDGF-AB was from R&D, Minneapolis, MN, USA.

Statistics

Summarized data are presented as means ± S.E.M. Statistical significance was calculated using ANOVA and Student's two-tailed *t*-test for unpaired comparisons with Bonferroni analysis for post hoc analysis as appropriate.

Results

Cell number and DNA synthesis

Cell proliferation was assessed by measuring cell number and DNA synthesis. Treatment with 1 and 10 mM DFMO reduced number of cells by about 20% (Fig. 1a). As seen in Fig. 1a, treatment with either the arginase inhibitor NOHA (100 μM) or the NO synthase inhibitor L-NAME (100 μM) for 5 days had no effect on cell number. However, a reduction in cell number by about 40% was observed if NOHA was administered in combination with 1 mM DFMO (Fig. 1a). The combination of DFMO and NOHA tended to have a more pronounced effect than treatment with DFMO alone. This difference was, however, not statistically significant. Treatment with 1 mM DFMO in the presence of putrescine (100 μM) had no effect on cell number (77,000 ± 13,100 cells/ml after treatment with DFMO + putrescine vs. 78,000 ± 21,600 cells/ml in untreated controls, $n = 3$ in each group). Protein concentration in cellular homogenates was not affected by the different treatments, suggesting that these treatments have no effect on cell viability (Fig. 1b). Treatment with DFMO (1 mM) for 24 h reduced FCS (5%) induced DNA synthesis, assessed by [^{3}H]-thymidine incorporation, by about 25% (30,699 ± 1,400 CPM/μg protein in DFMO treated cells vs. 41,570 ± 3,155 CPM/μg protein in control cells, $P < 0.05$, $n = 4$ in each group), while it had no effect on protein concentration (32.1 ± 2.1 μg/ml in DFMO treated vs. 34.2 ± 2.8 μg/ml in control cells, $n = 4$ in each group).

Effects of DFMO on cell morphology and cell viability

A7r5 cell morphology monitored by phase contrast microscopy and cell viability determined by Trypan blue exclusion test were not affected by treatment with DFMO, NOHA and L-NAME (not shown). No differences were detected in cell viability between the DFMO treated (1 and 10 mM for 2 days) and non-treated cell populations, determined via fluorescence labelling and cell-specific epifluorescence microscope analyses (Fig. 2). The visualisation of cell nuclei morphology and DNA fragmentation with DAPI and TUNEL, used separately and together, showed that DFMO (1 or 10 mM) did not induce apoptosis or increase the number of necrotic cells (Fig. 2). In both DFMO and non-DFMO treated cell populations TUNEL

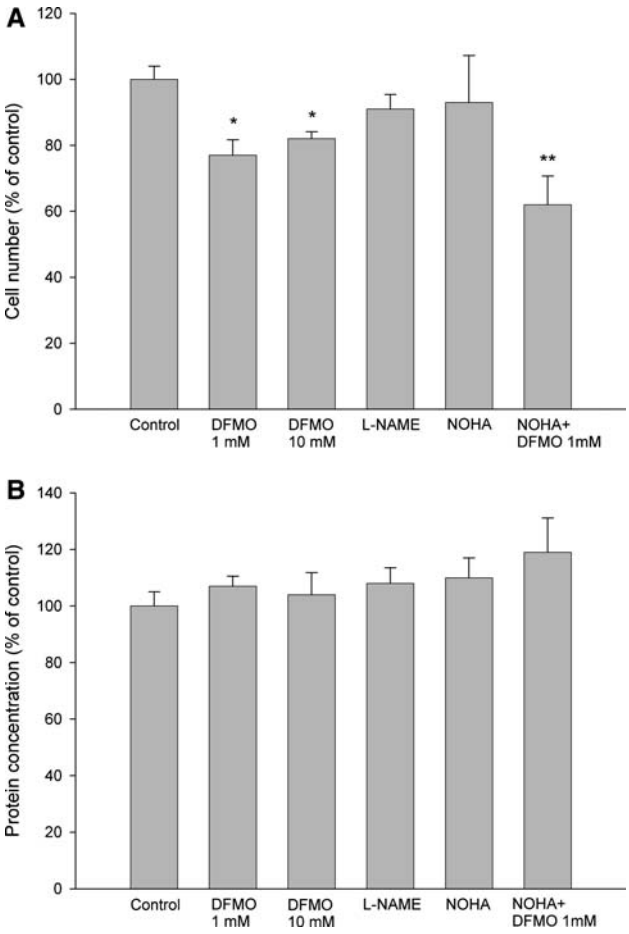

Fig. 1 Effects of treatment with or without DFMO (1 and 10 mM), L-NAME (100 μM), NOHA (100 μM) and NOHA (100 μM) + DFMO (1 mM) for 5 days on A7r5 cell number (**a**) and total cellular protein concentration (**b**). *, **$P < 0.05$ and 0.01, respectively, when compared to control. Values are presented as means ± SEM of 3–8 observations in each group

and DAPI labelling used together revealed only a few DNA strand breaks, comprising necrotic nuclei, DNA debris or dividing cells as demonstrated in Fig. 2. In addition, the cytoskeleton labelling (phalloidin conjugated with fluorophores binding to F-actin) did not reveal any differences in cell morphology between the DFMO and non-DFMO treated cell populations (Fig. 2), supporting that DFMO did not affect the viability of the A7r5 vascular smooth muscle cells.

Cellular polyamine concentration

Treatment with DFMO (1 mM) for 2 days reduced putrescine and spermidine concentrations by 70 and 74%, respectively, while it had no effect on spermine concentration (Fig. 3). Identical results were observed in response to 5 days' treatment with DFMO (data not shown). Treatment with NOHA (100 μM) alone for 1 or 5 days had no effect on putrescine concentration, while NOHA administered in combination with DFMO (1 mM) reduced putrescine to an undetectable level (Table 1). Spermidine concentration was not affected by treatment with NOHA alone, while NOHA + DFMO reduced spermidine by about 75% (Table 1). Spermine concentration was not affected by any of the different treatments (Table 1). Treatment with L-NAME had no effect on polyamine concentrations (Table 1). These data show that DFMO but not NOHA or L-NAME affects cellular levels of

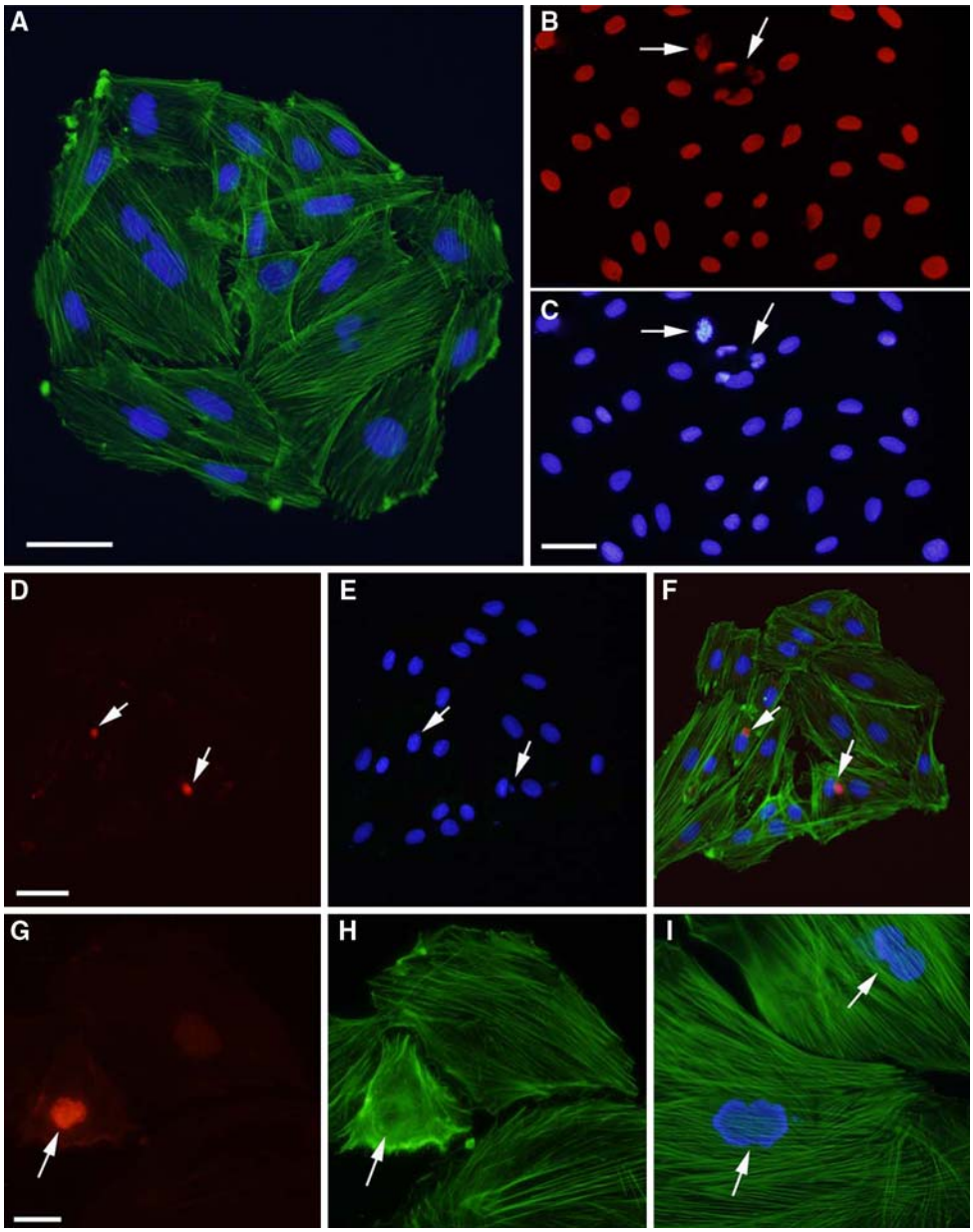

Fig. 2 Phalloidin staining for F-actin (*green*), DAPI staining for cell nuclei (*blue*) and TUNEL staining for apoptosis (*red*) show that DFMO treatment (1 mM for 2 days) has no effect on either A7r5 cell morphology or cell death and apoptosis. Identical results were observed in response to 10 mM DFMO (not shown). **a** DFMO treated cells in overlay with no TUNEL staining. **b** DNase treated cells for positive TUNEL staining control. **c** The same cells as in **b** stained with DAPI. *Arrows* in **b** and **c** show a pycnotic nucleus and DNA debris. **d–f** DFMO treated cells with TUNEL signal (*arrows*), which is not associated with nuclei. In very few non-treated and dying cells a TUNEL signal was detected (*arrows* in **g** and **h**). DFMO treated cells show high viability as demonstrated by a cell division (*arrows* in **i**). Slides from three independent experiments were analysed. *Scale bar* in **a** represents 50 μm. *Scale bars* in **c** and **d** represent 50 μm (for **b–f**). *Scale bar* in **g** represents 20 μm (for **g–i**)

polyamines and that identical effects of treatment with the polyamine synthesis inhibitors are observed within the time frame under study (1–5 days).

Flow cytometry and Western blotting for cyclin A

In order to examine the mechanism by which DFMO attenuates cell proliferation, cell cycle phase distribution was determined. DNA content in A7r5 cells treated with or without DFMO (1 mM) was analysed by flow cytometry of propidium iodide-stained cells. Cell cycle phase distribution was determined in about 13,000 nuclei in each sample. As seen in Fig. 4 treatment with DFMO for 2 days increased the number of cells in S phase by about 20% showing that DFMO causes an accumulation of cells in the S phase of the cell cycle. Cyclin A regulates the progression through the S phase of the cell cycle and we hypothesized that accumulation of cells in the S phase by DFMO treatment might be due to reduced cyclin A expression. In A7r5 cells treated for 2 days with 1 mM DFMO cyclin A expression was down-regulated by about 25% as determined by photodensitometric scanning of the immunospecific band and normalisation to the protein load in each lane (77 ± 3% in DFMO treated cells vs. 100% in control cells, $P < 0.01$, $n = 3$ and 4 in each group). A representative Western blot for cyclin A is shown in Fig. 5. A higher concentration (10 mM) of DFMO had a similar effect as 1 mM (Fig. 5).

Effects of DFMO on DNA synthesis in rat tail artery

In order to confirm the anti-proliferative effect of polyamine synthesis inhibition in whole vascular preparations, DNA synthesis was determined in endothelium-denuded rat tail arterial rings growth-stimulated with FCS (1%) + PDGF-AB (100 ng/ml) in the absence or presence of DFMO (5 mM) or NOHA (200 μM). In these experiments we used PDGF-AB as growth-promoter since it is known to stimulate DNA synthesis several fold in arterial rings (Lindqvist et al. 2001). Treatment with DFMO for 5 days reduced PDGF-AB-induced DNA synthesis in arterial rings by about 35%, while inhibition of arginase by NOHA had no effect (Fig. 6). In one set of experiments we determined the level of inhibition of PDGF-AB-induced DNA synthesis by DFMO. PDGF-AB increased DNA synthesis by about 30% in rat tail artery (276,439 ± 13,031 CPM/mg in PDGF-AB treated artery vs. 208,881 ± 22,165 CPM/mg in control artery, $n = 3$ in each group). DFMO fully reversed PDGF-AB-induced DNA synthesis (190,328 ± 34,395 CPM/mg in PDGF-AB + DFMO treated aorta vs. 208,881 ± 22,165 CPM/mg in control aorta, $n = 3$ in each group). These data show that DFMO reduces not only proliferation of cultured vascular smooth muscle cell line A7r5 cells but also fully prevents the proliferation of arterial smooth muscle cells present in their natural environment within the vascular wall.

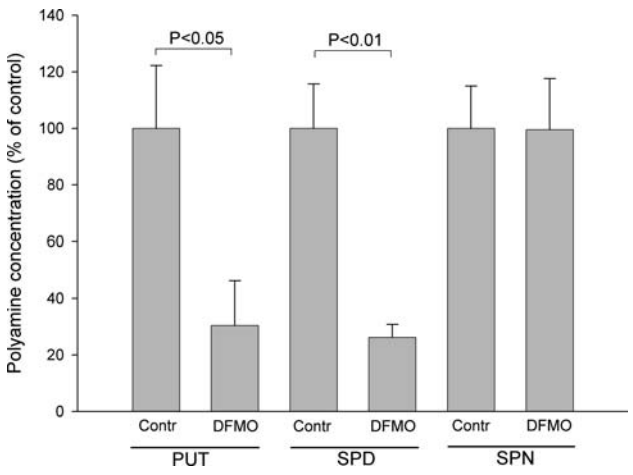

Fig. 3 Treatment with DFMO for 2 days attenuates A7r5 cell putrescine (*PUT*) and spermidine (*SPD*) but not spermine (*SPN*) concentration. Values are presented as means ± SEM of 4 observations in each group

Western blotting for eNOS and arginase I

A7r5 cells have been reported to express eNOS but not iNOS or nNOS (Moneer et al. 2003). We blotted for eNOS to ensure that our batches of A7r5 cells also expressed this protein. As demonstrated in Fig. 7 (panel A) A7r5 cells expressed a strong band at 140 kDa corresponding to the correct size of the eNOS protein. We determined arginase I protein expression in A7r5 cells, endothelium-denuded rat tail artery and rat aorta demonstrating lack of arginase I protein in A7r5 cells, rat tail artery and in aorta (Fig. 7b). Also after treatment of rat aorta with PDGF-AB for 5 days no arginase I protein was observed (data not shown).

Discussion

Here, we show that polyamine synthesis inhibition by treatment with the ODC inhibitor DFMO attenuates proliferation of vascular smooth muscle cells by reducing DNA synthesis and slowing the progression through S phase, thereby causing an accumulation of cells in the S phase of the cell cycle. The anti-proliferative effect of DFMO was observed not only in cultured A7r5 vascular smooth muscle cells, confirming data by Ignarro et al. (2001), but also in arterial rings. Thus, our data show that DFMO reduces proliferation not only in cultured vascular smooth muscle cells but also in vascular smooth muscle cells present in their natural environment. In the present study, we provide further insight into the mechanism behind the anti-proliferative

Table 1 Putrescine (PUT), spermidine (SPD) and spermine (SPN) concentrations in A7r5 cells treated with or without NOHA (100 μM), L-NAME (100 μM) and NOHA (100 μM) + DFMO (1 mM) for 5 days

	PUT (nmol/mg protein)	SPD (nmol/mg protein)	SPN (nmol/mg protein)
Control (5)	0.18 ± 0.05	1.95 ± 0.14	6.40 ± 0.64
NOHA (5)	0.21 ± 0.01	1.79 ± 0.27	5.79 ± 0.77
L-NAME (3)	0.12 ± 0.06	1.44 ± 0.62	5.37 ± 0.33
NOHA + DFMO (4)	0 ± 0*	0.50 ± 0.05***	5.10 ± 0.65

Number of determinations are given within brackets. Values are presented as means ± SEM

* $P < 0.05$, *** $P < 0.001$ as compared to control values

effect of DFMO by showing that this agent causes cell cycle arrest in the S phase probably via attenuation of cyclin A expression. Accumulation of cells in the S phase in response to treatment with DFMO has been reported in human prostatic epithelial cells and CHO cells, whereas other effects, such as arrest in G0/G1 and G2, have been reported in different cell systems (Anehus et al. 1984; Oredsson 2003; Scorcioni et al. 2001), suggesting that polyamine synthesis inhibition affects the cell cycle in a cell-type specific manner. Our data suggest that the mechanism by which polyamine synthesis inhibition causes accumulation of vascular smooth muscle cells in the S phase involves reduced expression of cyclin A. Lowered expression of cyclin A reduces the progression through S and probably the A7r5 cells passes over into G2 but at low speed. In the S phase, nuclei cyclin A is proposed to regulate the DNA replication via participating in the assembly, activation and regulation of the replication structure (Oredsson 2003). We suggest that reduction of cyclin A expression is a mechanism behind the accumulation of vascular smooth muscle cells in the S phase, but polyamine synthesis inhibition may also affect expression and activities of cyclin-dependent kinases (CDKs), CDK inhibitors and p53. Polyamine depletion has been reported to induce the CDK inhibitor p21 in breast cancer cells (Hu et al. 2005) and to stabilise p53 mRNA in intestinal epithelial cells (Zou et al. 2006).

We observed no effects of DFMO on vascular smooth muscle cell morphology and cell viability, suggesting that this compound acts specifically on DNA synthesis and cell cycle progression without any unspecific cytotoxic effects. The main effect of DFMO has been reported to be cytostatic and not cytotoxic which is in line with our observations (Flamigni et al. 2007). Several reports suggest that polyamines may affect apoptosis but the relationship seems complex and dependent on, e.g. cell type, polyamine levels and death stimulus (Flamigni et al. 2007). Here, we show that DFMO treatment does not induce cell death in vascular smooth muscle cells emphasizing that DFMO acts via cytostatic rather than cytotoxic mechanisms.

DFMO is a potent and specific irreversible inhibitor of ODC. It has been widely used in the 1–5 mM concentration range to inhibit cell proliferation in cancer cell lines (Pegg et al. 1995b). In the present study we confirm the polyamine synthesis inhibition by DFMO by measuring the cellular polyamine levels with HPLC. Both putrescine and spermidine are significantly reduced by DFMO while spermine is unaffected at all time points under study, suggesting that the inhibition of DNA synthesis and the accumulation of vascular smooth muscle cells in S phase of the cell cycle are specific effects of lowered putrescine and spermidine. These data implicate that putrescine and spermidine but not spermine regulates cell cycle progression and proliferation of vascular smooth muscle cells.

Vascular wall inflammation is an early event in atherosclerosis (Ross 1993; Libby 2002) and inflammation promoters such as LPS and interleukins induce arginase I (Wei et al. 2000; Sonoki et al. 1997; Salimuddin et al. 1999). Here we show that rat aorta, rat tail artery and A7r5 cells, not exposed to inflammation promoters, lack arginase I protein, suggesting that the lack of effect of NOHA in A7r5 cells and rat artery is due to absence of arginase I activity. NOHA has been reported to reduce polyamines and DNA synthesis in a vascular smooth muscle cell line obtained from rat aorta expressing arginase I but not arginase II mRNA and protein (Ignarro et al. 2001; Wei et al. 2000). Durante et al. (2001) and Zhang et al. (2001) have shown that arginase I is the predominant vascular isoform of arginase. Taken together these data show that arginase I expression, and thus the response to arginase inhibition, depends on vascular smooth muscle cell strain and on stimulation with inflammation promoters and cytokines. Cell-type specific differences in arginase activity have been reported also in different human breast cancer cell lines (Singh et al. 2001). These authors demonstrate that NOHA reduces cell proliferation in human breast cancer cell lines with high arginase activity, while it has no effect in cells with low arginase activity. NOHA inhibits arginase activity in Caco-2 tumour cells with IC_{50} around 1 μM (Buga et al. 1998) supporting that the NOHA concentration used in the present study (100–200 μM) is sufficient to fully block any possible isoform of arginase activity both in cultured vascular smooth muscle cells and

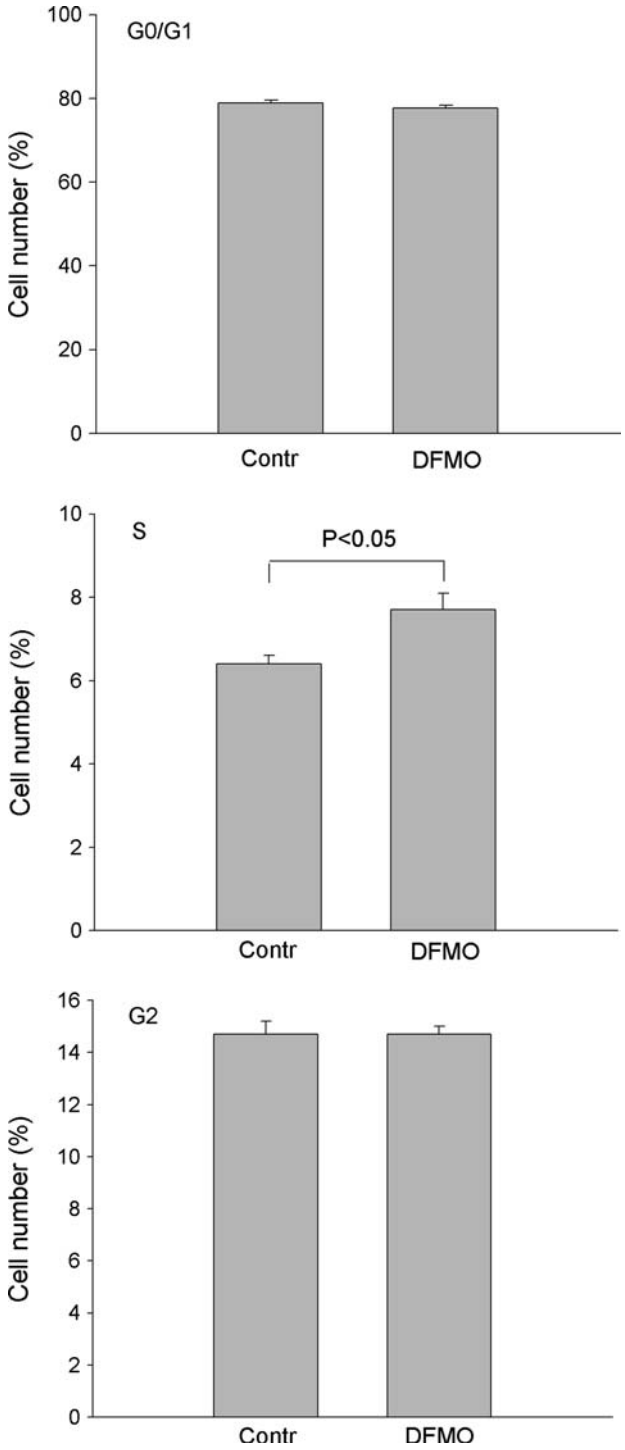

Fig. 4 Cell cycle phase distribution in A7r5 cells treated with or without DFMO (1 mM) for 2 days. DNA was stained with propidium iodide and the distribution of cells in G0/G1, S and G2 phases computed based on DNA histograms and presented as percentage. For each sample about 13,000 nuclei were analysed. Values are presented as means ± SEM of 3–5 observations in each group

in whole vascular tissue. Additionally our experiments were performed in low-arginine-containing medium to reduce the substrate concentration for arginase.

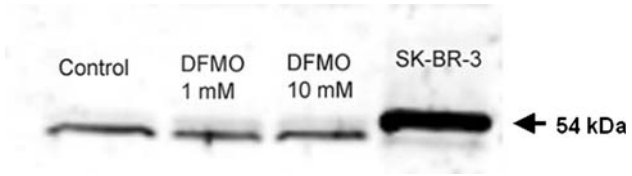

Fig. 5 Western blot for cyclin A. The A7r5 cells expressed a strong band at 54 kDa corresponding to the correct size of the cyclin A protein. Human breast cancer SK-BR-3 cells (ATCC, Manassas, VA, USA) were used as positive control as recommended. Treatment with DFMO (1 and 10 mM) reduces cyclin A expression by about 25% (see text for summarized data). This blot represents one experiment out of three

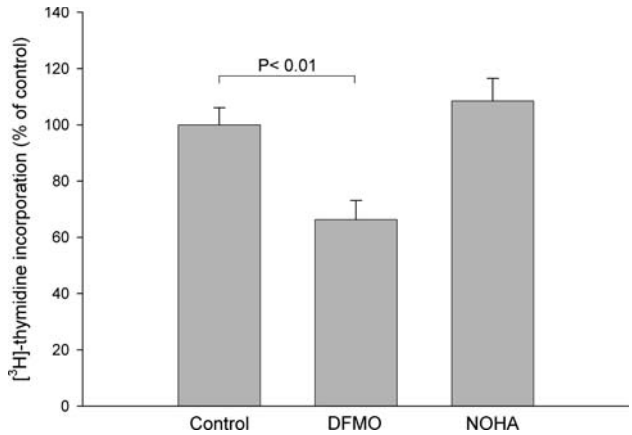

Fig. 6 Treatment with DFMO (5 mM) for 5 days reduces PDGF-AB (100 ng/ml) induced vascular smooth muscle cell proliferation, assessed by DNA synthesis measurement using [^{3}H]-thymidine incorporation, in rat tail arterial rings. Arginase inhibition by NOHA (200 μM) had no effect. Values are presented as means ± SEM of 4–6 observations in each group

Buga et al. (1998) and Ignarro et al. (2001) have reported that NO donor agents inhibit tumour cell and vascular smooth muscle cell proliferation by reducing ODC activity. Here, we report that the NOS inhibitor L-NAME, used in appropriate concentration (100 μM, Pfeiffer et al. 1996), lacks effect on A7r5 vascular smooth muscle cell proliferation. The A7r5 cells express the eNOS protein and thus the lack of effect of L-NAME is not due to absence of eNOS expression. One possibility is, however, that although we inhibit eNOS activity by L-NAME the amount of NO produced by the A7r5 cells is too small to affect cell proliferation.

Proliferating cells need high amounts of polyamines, and ODC is induced in response to this demand (Pegg and McCann 1982; Pegg et al. 1995b). Vascular smooth muscle cells, stimulated to proliferate by PDGF-AB as occurs in atherosclerosis and restenosis, have probably higher ODC activity and polyamine levels than quiescent vascular smooth muscle cells. In the present study, we show that the ODC inhibitor DFMO reduces polyamines and attenuates

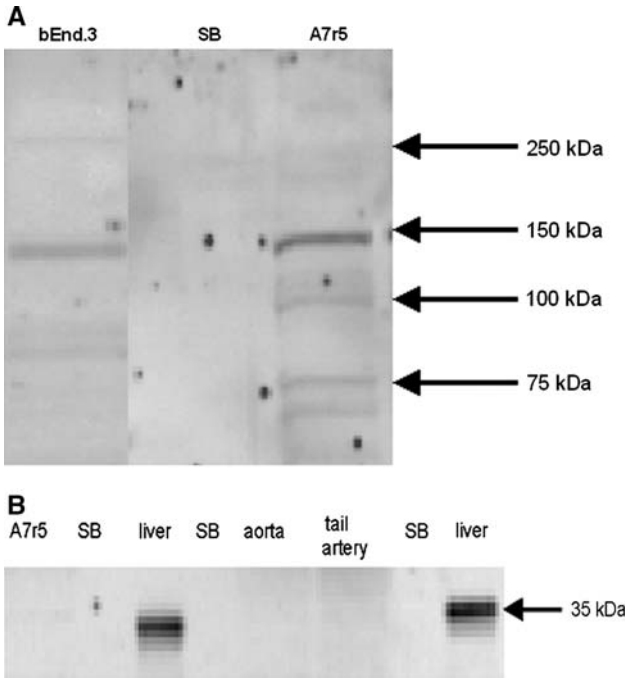

Fig. 7 Western blot showing expression of eNOS (140 kDa) protein in A7r5 cells (**a**) and lack of arginase I protein (35 kDa) expression in A7r5 cells, rat aorta and rat tail artery (**b**). The A7r5 cells expressed a strong band at 140 kDa corresponding to the correct size of the eNOS protein. Endothelial cell lysate (bEnd.3 cells, ATCC), expressing a strong band at 140 kDa, and rat liver, expressing a strong band at 35 kDa, corresponding to the correct size of the eNOS and arginase I proteins, were used as positive controls as recommended. Empty lanes were loaded with sample buffer (*SB*). These blots represent one experiment out of two for eNOS and one experiment out of four for arginase I

proliferation not only in cultured vascular smooth muscle cells but also in PDGF-AB-stimulated arterial tissue. These data suggest that DFMO, acting by reducing DNA synthesis and accumulating the cells in S phase of the cell cycle, can be used to prevent unwanted vascular smooth muscle cell proliferation occurring in atherosclerosis and restenosis.

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