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Growth-inhibitory effect of cyclic GMP- and cyclic AMP-dependent vasodilators on rat vascular smooth muscle cells: effect on cell cycle and cyclin expression

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1 The possibility that the antiproliferative effect of cyclic GMP- and cyclic AMP-dependent vasodilators involves an impaired progression of vascular smooth muscle cells (VSMC) through the cell cycle and expression of cyclins, which in association with the cyclin-dependent kinases control the transition between the distinct phases of the cell cycle, was examined.

2 FCS (10%) stimulated the transition of quiescent VSMC from the G0/G1 to the S phase (maximum within 18–24 h and then to the G2/M phase (maximum within 22–28 h). Sodium nitroprusside and 8-Br-cyclic GMP, as well as forskolin and 8-Br-cyclic AMP markedly reduced the percentage of cells in the S phase after FCS stimulation.

3 FCS stimulated the low basal protein expression of cyclin D1 (maximum within 8–24 h) and E (maximum within 8–38 h) and of cyclin A (maximum within 14–30 h). The stimulatory effect of FCS on cyclin D1 and A expression was inhibited, but that of cyclin E was only minimally affected by the vasodilators.

4 FCS increased the low basal level of cyclin D1 mRNA after a lag phase of 2 h and that of cyclin A after 12 h. The vasodilators significantly reduced the FCS-stimulated expression of cyclin D1 and A mRNA.

5 These findings indicate that cyclic GMP- and cyclic AMP-dependent vasodilators inhibit the proliferation of VSMC by preventing the progression of the cell cycle from the G0/G1 into the S phase, an effect which can be attributed to the impaired expression of cyclin D1 and A.

Keywords: VSMC; proliferation; nitric oxide donor; sodium nitroprusside; forskolin; 8-Br-cyclic GMP; 8-Br-cyclic AMP; cell cycle; cyclins

Abbreviations: CAS 92-4678, 4-phenyl-furoxan-3-carboxylic acid [pyridyl-3-yl-methyl]-amide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis(β-aminoethyl ether)-tetraacetic acid; FCS, foetal calf serum; HEPES, N-2-hydroxyethyl piperazine-N-2-ethanesulphonic acid; MOPS, 3-(N-morpholino)-propanesulphonic acid; NO, nitric oxide; NOS II, inducible nitric oxide synthase; NOS III, endothelial nitric oxide synthase; SDS, sodium dodecylsulphate; SNAP, S-nitroso-N-acetylpenicillamine; SNP, sodium nitroprusside; VSMC, vascular smooth muscle cells

Introduction

Endothelium-derived nitric oxide (NO) plays a pivotal role in the control of vascular homeostasis by regulating vascular tone (Furchgott & Vanhoutte, 1989) and the interaction of blood cells with the vascular wall (Radomski *et al*., 1987). In addition, NO may also contribute to the maintenance of vascular smooth muscle cells (VSMC) in a non-proliferative state. Such a concept is supported by several lines of evidence. First NO donors, as wells as NO generated in VSMC following gene transfer of endothelial NO synthase (NOS III), or the cytokine-induced expression of inducible NO synthase (NOS II), strongly inhibited the growth of cultured VSMC (Garg & Hassid, 1989; Kariya *et al*., 1989; Scott-Burden *et al*., 1992; Kullo *et al*., 1997). Second, administration of NO donors reduced the development of intimal thickening in rat (Guo *et al*., 1994) and porcine carotid (Groves *et al*., 1995), and rabbit femoral arteries injured by a balloon catheter (Marks *et al*., 1995), and also in humans after percutaneous transluminal angioplasty (The ACCORD Study Investigators, 1997). Third, administration of L-arginine, the substrate of NOS, to rabbits attenuated intimal thickening of aortae in response to balloon injury (McNamara *et al*., 1993) or to a high cholesterol diet (Cooke *et al*., 1992), whereas NOS inhibitors exacerbated the accumulation of VSMC in the neointima (Cayatte *et al*., 1994). Furthermore, *in vivo* gene transfer of NOS III in rat carotid arteries prevented neointimal hyperplasia (Von der Leyen *et al*., 1995).

The growth-inhibitory effect of NO donors seems to be mediated, at least in part, by a cyclic GMP-dependent effector pathway, since VSMC proliferation is also impaired by a lipophilic analogue of cyclic GMP (Garg & Hassid, 1989; Assender *et al*., 1992), by an NO-independent activator of soluble guanylyl cyclase (Yu *et al*., 1995) and by activators of the particulate guanylyl cyclase (Abell *et al*., 1989). Although cyclic GMP may directly interfere with growth-related events, it is also conceivable that a cross-talk exists between cyclic GMP- and cyclic AMP-mediated signaling pathways since high concentrations of cyclic GMP have been reported to inhibit the degradation of cyclic AMP by phosphodiesterase III (Beavo & Reifsynder, 1990) and activate protein kinase A

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(Forte *et al*., 1992; Cornwell *et al*., 1994). Consistent with such a hypothesis it has been shown, that agents which elevate cyclic AMP, including prostaglandin E1 (Loesberg *et al*., 1985), adenosine (Jonzon *et al*., 1985; Loesberg *et al*., 1985), forskolin (Assender *et al*., 1992), or 8-Br-cyclic AMP (Jonzon *et al*., 1985; Assender *et al*., 1992) potently inhibited VSMC proliferation. Since the antiproliferative effect of NO donors or 8-Br-cyclic GMP is still observed when added up to 12 h after foetal calf serum (FCS) stimulation of growth-arrested VSMC (Kariya *et al*., 1989; Sarkar *et al*., 1997) it is conceivable that these vasodilators target growth-related events essential for the progression of the cell cycle from the G0/G1 into the S phase. NO may also inhibit growth-related events in the S phase and G2/M phase of the cell cycle since S-nitroso-N-acetylpenicillamine (SNAP) reduced DNA synthesis when added to VSMC synchronized in S phase (Sarkar *et al*., 1997). Furthermore, NO produced following induction of NOS II blocked macrophage-like cells in the early G2/M phase of the cell cycle (Takagi *et al*., 1994). These findings suggest that NO can interfere with the cell cycle progression, resulting in a growth arrest in defined phases. Since the sequential expression of cyclins, regulatory subunits of the cyclin-dependent kinases (Pines, 1995), is known to be determinant in the transition between the different phases of the cell cycle, it is conceivable that NO might exert its antiproliferative effect through a modulation of cyclin expression. Therefore the aim of the present study was to assess the effect of NO donors on the expression of cyclins and to better characterize the effect of cyclic GMP- and cyclic AMP-dependent vasodilators on the cell cycle of VSMC.

Methods

Materials

SNP was obtained from Merck (Darmstadt, Germany), forskolin and 8-Br-cyclic GMP from Fluka (Deisenhofen, Germany), and 8-Br-cyclic AMP from Biomol (Hamburg, Germany). CAS 92-4678 (4-phenyl-furoxan-3-carboxylic acid [pyridyl-3-yl-methyl]-amide) was kindly supplied by Hoechst (Frankfurt, Germany). Minimum essential medium containing Earle's salts, trypsin, penicillin and streptomycin were supplied by Gibco BRL (Eggenstein, Germany) and FCS by Biochrom (Berlin, Germany). All plastic-ware was purchased from Falcon (Becton Dickinson) and [α-^{32}P]-dCTP (3000 Ci m-mol^{-1}) from Hartmann Analytic (Braunschweig, Germany). All other chemicals were from Sigma (Deisenhofen, Germany). The *Eco*RI restriction fragment of human cyclin A was kindly provided by Dr T. Hunter (Salk Institute, La Jolla, U.S.A.) and the *Eco*RI restriction fragment of mouse cyclin D1 by Dr C.J. Sherr (St. Jude Children's Res. Hosp. Memphis, U.S.A.).

Cell culture

VSMC were isolated by elastase and collagenase digestion of thoracic aortae from male Wistar rats (250–300 g). Cells were cultured in minimum essential medium containing Earle's salts, 2 mM glutamine, 5 mM *N*-tris (hydroxymethyl)-methyl-2-aminoethanesulfonic acid NaOH, 5 mM N-2-hydroxyethyl-piperazine-N-2-ethanesulphonic acid (HEPES)-NaOH, (both at pH 7.3), 50 U ml^{-1} penicillin, 50 μg ml^{-1} streptomycin, and FCS (10%, v/v). Confluent cultures of VSMC were serially passaged with 0.05% trypsin-0.02% ethylenediaminetetraacetic acid (EDTA). All experiments were performed with confluent VSMC at passage 7–24. Cells were plated on either 24-well plates (growth studies) or on Petri dishes (35 mm diameter for flow cytometric analysis, 100 mm for Northern and Western blot analyses). Prior to the exposure of VSMC to the cyclic GMP- and cyclic AMP-dependent vasodilators, cells were incubated in fresh FCS-free medium containing 0.1% fatty acid-free bovine serum albumin every 24-h period for 2 days before treatment or as stated.

Cell growth studies

VSMC were seeded at approximately 70,000 cells/well in culture medium containing FCS (10%). After 24 h the culture medium was replaced with medium containing FCS (5%) prior to the addition of either SNP, the furoxan derivative CAS 92-4678 or forskolin. In the case of SNP, the medium containing the compound was replaced every 24 h. Cell number determinations were performed in triplicate, both 24 h after initial plating and 3 days after treatment, with a CASY-Coulter counter (Schaerfe System, Reutlingen, Germany).

Flow cytometric analysis

After a 48-h incubation period in FCS-free medium, cells were left either untreated or were exposed first to either a cyclic GMP- or cyclic AMP-dependent vasodilator and 5 min later to FCS (10%) and then incubated for the indicated time periods. The 5-min treatment period with the vasodilators has been shown previously to be associated with maximal production of cyclic GMP (Gruetter *et al*., 1981) and cyclic AMP (Leitman *et al*., 1986), respectively. At the appropriate times VSMC were trypsinized, centrifuged at $200 \times g$, washed with HEPES-buffered Tyrode's solution (pH 7.4), and then analysed for their DNA content by staining with propidium iodide according to the procedure of a commercial kit (Cycle TEST PLUS, Becton Dickinson, San Jose, CA, U.S.A.). Flow cytometric analysis was carried out using a FACScan (Becton Dickinson). DNA histogram analysis was performed using the ModFitLT software (Becton Dickinson).

Western blot analysis

VSMC were exposed to the cyclic GMP- and cyclic AMP-dependent vasodilators for 5 min prior to the addition of FCS (10%). Cells were then washed twice with cold HEPES-buffered Tyrode's solution (pH 7.4), harvested by scraping, and collected by centrifugation at $900 \times g$ for 3 min at 4°C. The cell pellets were incubated in 300 μl of buffer A (mM): HEPES pH 7.9, KCl 10, EDTA 0.1, ethyleneglycol-bis(β-aminoethyl ether)-tetra-acetic acid (EGTA) 0.1, dithiothreitol 1, phenylmethylsulfonyl fluoride 0.5, and 2 μg ml^{-1} each of leupeptin, pepstatin A, chymostatin, antipain, aprotinin, and trypsin inhibitor for 15 min at 4°C. The crude nuclei were obtained following lysis with Nonidet P-40 (0.6%) and collected by centrifugation at $15{,}000 \times g$ for 1 min at 4°C. The nuclear pellets were resuspended in 100 μl of buffer B (HEPES pH 7.9 20 mM, NaCl 0.4 M, EDTA 1 mM, EGTA 1 mM, dithiothreitol 1 mM and 2 μg ml^{-1} each of the protease inhibitors). Nuclei were shaken for 15 min and clarified by centrifugation at $15{,}000 \times g$ for 5 min at 4°C. The nuclear extracts (30–50 μg) were subjected to sodium dodecylsulphate (SDS) polyacrylamide gel (12%) electrophoresis and the separated proteins were then transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). A Ponceau staining was performed to ensure equal amounts of protein in each lane. The immobilized cyclin D1, E and A proteins were detected by subsequent incubation with polyclonal rabbit antibodies directed against cyclin D1, E and A,

respectively, (dilution 1 : 1000, overnight at 4°C; Santa Cruz, Heidelberg, Germany) and then with a secondary polyclonal donkey anti-rabbit immunoglobulin antibody conjugated to horseradish peroxidase (dilution 1 : 10,000, 1 h at 22°C; Calbiochem). The immunocomplexes were visualized using an enhanced horseradish peroxidase/luminol chemoluminescence reaction. Prestained molecular-mass markers were used as standards for SDS-polyacrylamide gel immunoblot analysis. The autoradiographs were analysed by scanning densitometry (Pharmacia densitometer equipped with a Kappa CCD video camera and the ImageMaster software).

Northern blot analysis

Total cellular RNA was prepared by acid guanidinium thiocyanate extraction and quantified by absorbance at 260 nm. Total RNA (20–30 μg) was size-fractionated by electrophoresis on 1.2% agarose gels containing 2.2 M formaldehyde in a buffer of 20 mM 3-(N-morpholino)-propanesulphonic acid (MOPS), 5 mM sodium acetate, and 1 mM EDTA pH 7.0. RNA was transferred to nylon membranes (Hybond N^+ Amersham Buchler, Braunschweig, Germany) and fixed by UV-crosslinking and baking at 80°C for 2 h. The membranes were prehybridized at 42°C for 4 h in a buffer containing 50% formaldehyde, 5 × Denhardt's solution, 5 × SSPE, 0.2% SDS, and 250 μg ml^{-1} denatured salmon sperm DNA. *Eco*RI restriction fragments for mouse cyclin D1 (1.3 kb) and human cyclin A (2.2 kb) were labelled with 30 μCi of α-^{32}P-dCTP (3000 Ci $mmol^{-1}$) using a labelling kit (Pharmacia, Freiburg, Germany). The [^{32}P]-DNA was purified by gel filtration (Nick columns, Pharmacia). Northern blots were hybridized in the same buffer as used for prehybridization at 42°C for 16–20 h with the labelled DNA. Thereafter the blots were washed twice for 30 min at 22°C in 6 × SSPE and 0.1% SDS, then twice for 15 min at 42°C in 1 × SSPE and 0.1% SDS and finally for 30 min at 55°C in 1 × SSPE and 0.1% SDS. After hybridization with the cyclin probes, filters were hybridized with a ^{32}P-labeled probe for 18S ribosomal RNA. Autoradiography was performed with Fuji RX film with intensifying screens (DuPont de Nemours, Bad Homburg, Germany) at −70°C. The autoradiographs were analysed by scanning densitometry. Cyclic mRNA levels were normalized to their respective 18S ribosomal mRNA levels and expressed in per cent of the signal obtained with FCS-stimulated VSMC.

DNA fragmentation

A photometric enzyme immunoassay (Cell Death Detection ELISA, Boehringer Mannheim) was used for the detection of DNA fragmentation of the VSMC. Cells were incubated with the cyclic GMP- and cyclic AMP-dependent vasodilators for 5 min prior to the addition of FCS (10%). After an incubation period of 22 h, cells were harvested by scraping, centrifuged at 700 × *g* for 10 min, washed with phosphate-buffered saline and resuspended in incubation buffer. The nucleosomes contained in the sample bind *via* their histone components to a mouse anti-histone antibody and the DNA-parts of the nucleosomes are linked to a mouse anti-DNA-peroxidase antibody. The amount of peroxidase bound in the immunocomplex was determined photometrically at 405 nm with 2,2′-azino-di-(3-ethylbenzthiazoline sulphonate) as a substrate.

Statistical analysis

Results are shown as mean ± s.e.mean of *n* experiments with different batches of VSMC. Statistical analyses were performed with Student's *t*-test or when more than two treatments were compared with one-way analysis of variance (ANOVA) followed by a Bonferroni *t*-test. A value $P<0.05$ was considered statistically significant.

Results

Effect of cyclic GMP- and cyclic AMP-dependent vasodilators on the FCS-induced proliferation of VSMC

Exposure of VSMC to FCS (5%) induced about a 4 to 5 fold increase in cell number after a 3-day incubation period (Figure 1). This FCS-stimulated cell growth was attenuated by the NO donors SNP (Figure 1a) and CAS 92 92-4678 (10 μM, reduction by 34%, $n=3$), as well as by forskolin (Figure 1b).

To exclude the possibility that the antiproliferative effects of the agents tested were due to cytotoxicity, morphological appearance of the cells was assessed by light microscopy and DNA fragmentation by a photometric enzyme immunoassay. Exposure of VSMC to either a cyclic GMP- or a cyclic AMP-dependent vasodilator for 22 h did neither induce any major changes in cell shape nor provoke DNA fragmentation compared to control cells (Table 1). However, exposure of

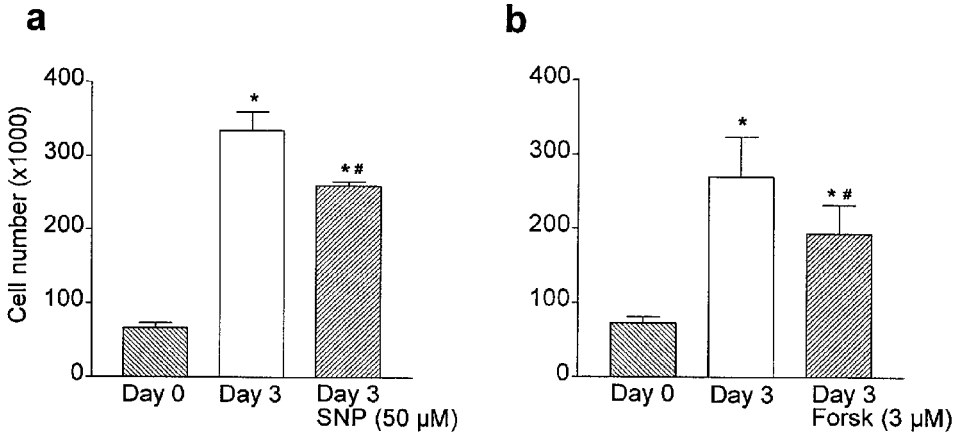

Figure 1 Effect of (a) SNP and (b) forskolin (Forsk) on the FCS (5%)-stimulated proliferation of cultured rat aortic smooth muscle cells. Cell numbers were determined 24 h after initial plating (Day 0) and 3 days after exposure of VSMC to FCS (Day 3) in the absence or presence of either SNP or forskolin. Data shown as mean ± s.e.mean of seven experiments for SNP and five experiments for forskolin. (*$P<0.05$ vs Day 0, #$P<0.05$ vs Day 3).

VSMC to a 10 fold higher concentration of SNP (3 mM) induced an about 11 fold increase in DNA fragmentation in two independent experiments.

Effect of cyclic GMP- and cyclic AMP-dependent vasodilators on the cell cycle of VSMC

Flow cytometric analysis after 48 h of FCS starvation revealed that more than 90% of VSMC were arrested in the G0/G1 phase (Figure 2). About 14 h after exposure to FCS (10%), VSMC progressed from the G0/G1 phase of the cell cycle into the S phase, and then after about 18 h into the G2/M phase (Figure 2). The effect of cyclic GMP- and cyclic AMP-dependent vasodilators on the transition of VSMC from the G0/G1 phase into the S phase was assessed at various time points after FCS stimulation. Both SNP and forskolin significantly prevented the FCS-stimulated transition of VSMC from the G0/G1 phase into the S phase (Figure 2a and b), but affected minimally the percentage of VSMC in the G2/M phase (Figure 2c). The inhibitory effect of SNP was concentration-dependent with a maximal reduction of $43\pm5\%$ (300 μM, Figure 3a). In addition to SNP, the NO donor CAS 92-4678 blocked S phase entry of VSMC with increasing concentrations with a maximal effect of $37\pm7\%$ (30 μM, $n=4$). Moreover, 8-Br-cyclic GMP, the membrane-permeable, stable analogue of cyclic GMP, reduced the amount of VSMC in the S phase by $39\pm10\%$ (3 mM, Figure 3b). Similar to the cyclic GMP-dependent vasodilators forskolin and 8-Br-cyclic

Table 1 Effect of cyclic GMP- and cyclic AMP-dependent vasodilators on the DNA fragmentation in cultured rat aortic smooth muscle cells exposed to FCS

Treatment	*DNA fragmentation* (% of FCS value)
Untreated	146 ± 87
FCS (10%, 22 h)	100
FCS + SNP (300 μM)	102 ± 25
FCS + 8-Br-cyclic GMP (3 mM)	90 ± 21
FCS + Forskolin (10 μM)	119 ± 17
FCS + 8-Br-cyclic AMP (0.3 mM)	83 ± 6

Results shown as mean $\pm$ s.e.mean of three experiments.

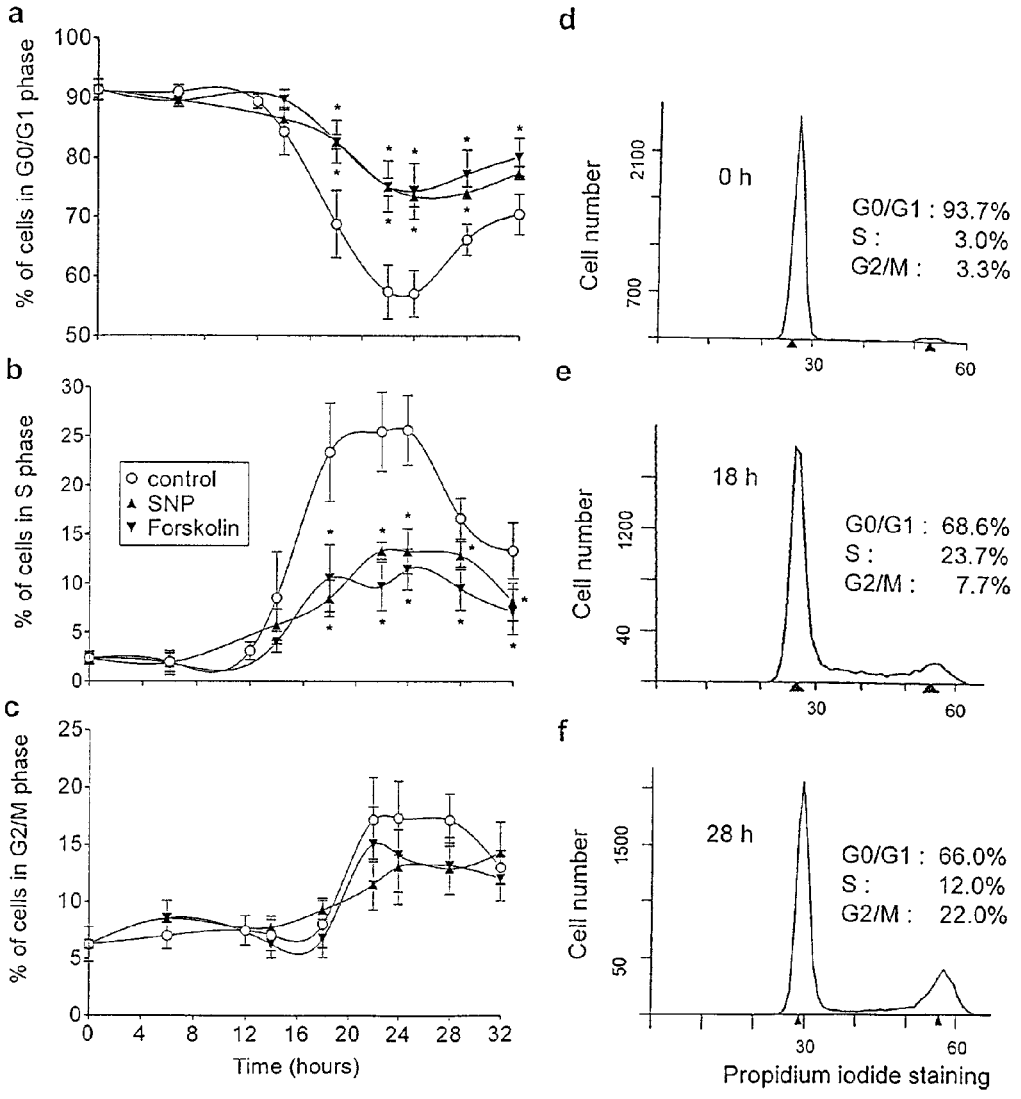

Figure 2 Flow cytometric analysis of cell cycle progression of cultured rat aortic smooth muscle cells. VSMC were arrested in the G0/G1 phase by FCS starvation for 48 h and then were stimulated to enter the cell cycle by the addition of FCS (10%). The effect of SNP (600 μM) and forskolin (3 μM) on the FCS-stimulated progression of VSMC through the cell cycle is also shown. In (a), (b) and (c) data shown as mean $\pm$ s.e.mean of five experiments for SNP and seven experiments for forskolin. In (d), (e) and (f) representative DNA histograms of VSMC at times 0, 18 and 28 h after FCS stimulation are depicted.

AMP markedly decreased the amount of VSMC in the S phase in a concentration-dependent manner with maximal reductions of 80±10% (10 μM, Figure 4a) and 67±4% (0.3 mM, Figure 4b), respectively.

Effect of cyclic GMP- and cyclic AMP-dependent vasodilators on the protein expression of cyclins

Low levels of the G1 cyclin D1 and cyclin E protein were found in quiescent VSMC, whereas S phase cyclin A protein levels were minimal or even below the detection limit (Figure 5). Exposure of VSMC to FCS (10%) caused a time-dependent increase first of cyclin D1 protein (maximum value within 8–24 h and cyclin E protein (maximum value within 8–38 h) and thereafter of cyclin A protein (maximum value within 14–30 h) (Figure 5). The effect of cyclic GMP- and cyclic AMP-dependent vasodilators on the FCS-induced expression of cyclin D1, E and A protein was determined after 22 h of treatment, a time point at which a significant and consistent expression of the three cyclins was observed. Both SNP and 8-Br-cyclic GMP significantly reduced the FCS-stimulated expression of cyclin D1 protein by about 70 and 42%, and cyclic A protein by about 42 and 36%, respectively, whereas the expression of cyclin E protein was only slightly, though significantly attenuated by SNP, but not by 8-Br-cyclic GMP (Figure 6). The cyclic AMP-dependent vasodilators forskolin and 8-Br-cyclic AMP significantly decreased the FCS-induced expression of cyclin D1 protein by about 82 and 77%, and cyclin A protein by about 80 and 79%, respectively (Figure 6). In contrast, the cyclic AMP-dependent vasodilators exerted only minimal effects on the FCS-induced cyclin E expression (Figure 6). The FCS-stimulated cyclin A protein expression was inhibited to a significantly greater extent by the cyclic AMP-dependent vasodilators than by the cyclic GMP-dependent vasodilators.

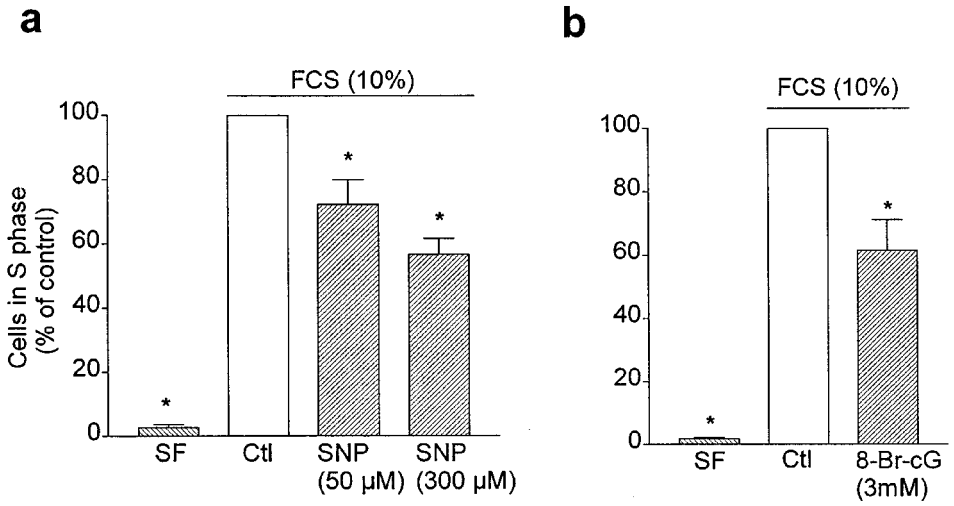

Figure 3 Effect of (a) SNP and (b) 8-Br-cyclic GMP (8-Br-cG) on the FCS-stimulated S phase entry of cultured rat aortic smooth muscle cells. Following growth arrest VSMC were stimulated to enter the cell cycle by the addition of FCS (10%) for 18 h. VSMC were exposed to a cyclic GMP-dependent vasodilator 5 min prior to FCS. Data shown as mean±s.e.mean of six experiments for SNP and three experiments for 8-Br-cyclic GMP. (*$P<0.05$ vs Ctl). Serum-free: SF; Control: Ctl.

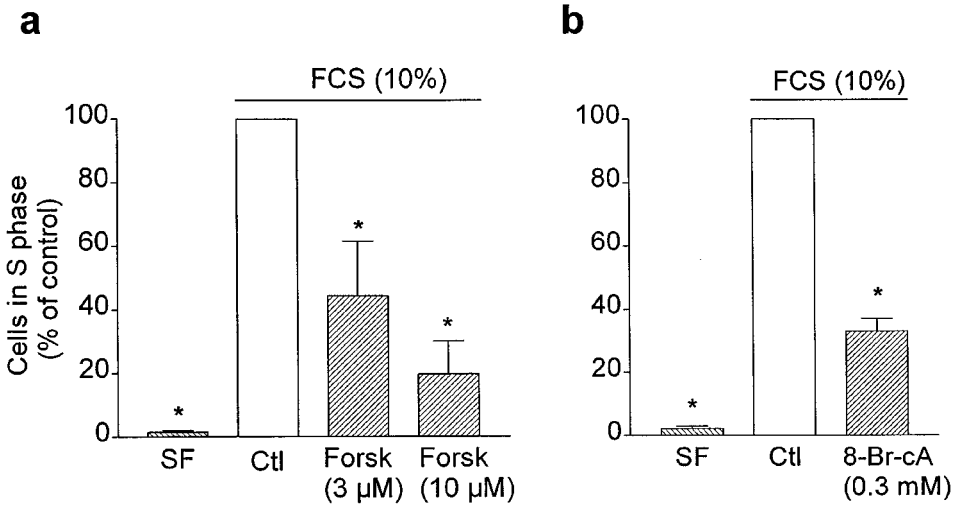

Figure 4 Effect of (a) forskolin (Forsk) and (b) 8-Br-cyclic AMP (8-Br-cA) on the FCS-stimulated S phase entry of cultured rat aortic smooth muscle cells. Following growth arrest VSMC were stimulated to enter the cell cycle by the addition of FCS (10%) for 18 h. VSMC were exposed to a cyclic AMP-dependent vasodilator 5 min prior to FCS. Data shown as mean±s.e.mean of three experiments for forskolin and five experiments for 8-Br-cyclic AMP. (*$P<0.05$ vs Ctl). Serum-free: SF; Control: Ctl.

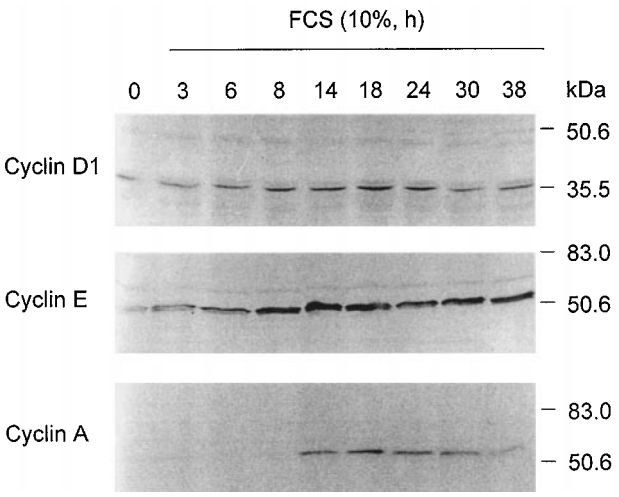

Figure 5 Representative Western blots showing the time course of cyclin D1, E, and A protein expression induced by FCS (10%) in cultured rat aortic smooth muscle cells. Equal amounts of protein from nuclear extracts were subjected to SDS–PAGE, transferred to nitrocellulose membranes, and immunoblotted with polyclonal antibodies against G1 phase cyclin D1 (top), and cyclin E (center) and S phase cyclin A (bottom). Similar results were obtained in two additional experiments.

Effect of cyclic GMP- and cyclic AMP-dependent vasodilators on the mRNA expression of cyclins

Low levels of cyclin D1 and cyclin A mRNA were detectable in FCS-deprived VSMC (Figure 7). Exposure of VSMC to FCS (10%) increased the expression of cyclin D1 mRNA after a lag-phase of about 2 h, and thereafter the cyclin D1 mRNA levels remained elevated for the next 28 h (Figure 7a). Cyclin A mRNA increased gradually after a delay of about 12 h to reach a maximum level at about 26 h (Figure 7b). Both SNP and 8-Br-cyclic GMP significantly reduced the FCS-stimulated expression of cyclin D1 mRNA by about 54 and 57% (Figure 8a) and cyclin A mRNA by about 44 and 74% (Figure 8b) as assessed after 16 h. Forskolin abolished the FCS-induced expression of cyclin D1 and A mRNA (Figure 8a and b) and 8-Br-cyclic AMP abolished the FCS-induced expression of cyclin D1 mRNA, whereas cyclin A mRNA was inhibited by about 75%, as assessed after 16 h.

Discussion

The present findings demonstrate that SNP and forskolin inhibit the proliferation of VSMC by preventing the progression of the cell cycle from the G0/G1 phase into the S phase. This cell cycle arrest seems to be due to an impaired expression of the G1 phase cyclin D1 and early S phase cyclin A *via* cyclic GMP- and cyclic AMP-dependent mechanisms.

Previous studies have shown that both NO-dependent (SNP) (Garg & Hassid, 1989; Kariya *et al*., 1989; Sarkar *et al*., 1997), and NO-independent (the indazole derivative YC-1) (Yu *et al*., 1995) activators of soluble guanylyl cyclase, as well as activators of the particulate enzyme (atrial natriuretic peptide) (Abell *et al*., 1989) inhibited the serum-stimulated [^{3}H]-thymidine incorporation when added up to 12 h after serum stimulation of quiescent VSMC. Such an inhibitory effect of cyclic GMP-dependent vasodilators suggests that VSMC were growth-arrested in the G0/G1 phase of the cell cycle. In the present study, flow cytometric analysis provided

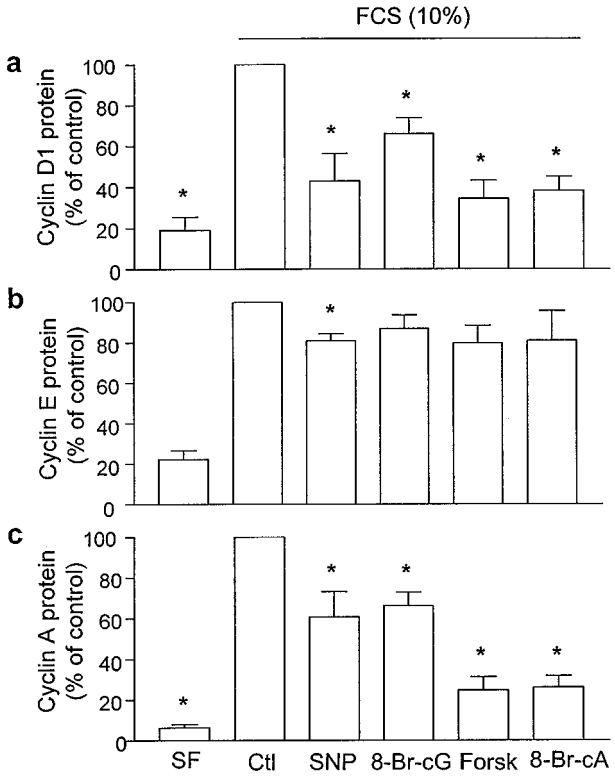

Figure 6 Cumulative data from Western blots showing the effect of cyclic GMP- and cyclic AMP-dependent vasodilators on the FCS-induced (a) cyclin D1, (b) cyclin E and (c) cyclin A protein expression in cultured rat aortic smooth muscle cells. VSMC were exposed to FCS (10%) for 22 h. The vasodilators were added 5 min prior to FCS. Results shown as mean ± s.e.mean of at least four experiments. (*$P<0.05$ vs Ctl). Serum-free: SF; control: Ctl; SNP: 300 μM; 8-Br-cyclic GMP: 8-Br-cG, 3 mM; forskolin: Forsk, 10 μM; 8-Br-cyclic AMP: 8-Br-cA, 0.3 mM.

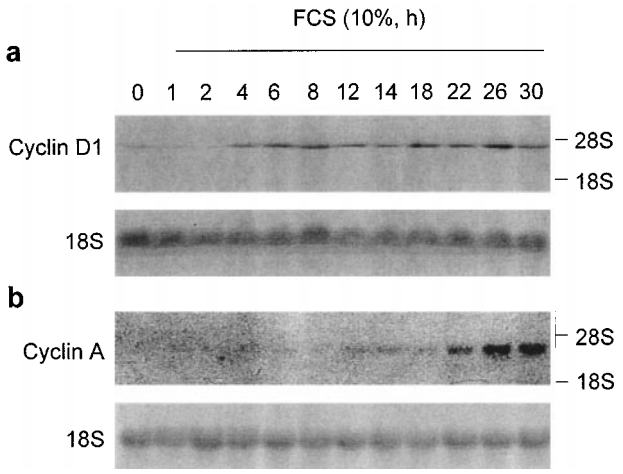

Figure 7 Representative Northern blots showing the time course of cyclin D1 and cyclin A mRNA expression induced by FCS (10%) in cultured rat aortic smooth muscle cells. (a) cyclin D1 cDNA probe (top), 18S ribosomal RNA (bottom), (b) cyclin A cDNA probe (top), 18S ribosomal RNA (bottom). Similar results were obtained in two additional experiments for cyclin D1 and in an additional experiment for cyclin A.

further evidence for this assumption. Since the inhibitory effect of the cyclic GMP-dependent vasodilators was mimicked by 8-Br-cyclic GMP, the membrane-permeable, stable analogue of cyclic GMP, it is likely, that the cyclic GMP effector pathway

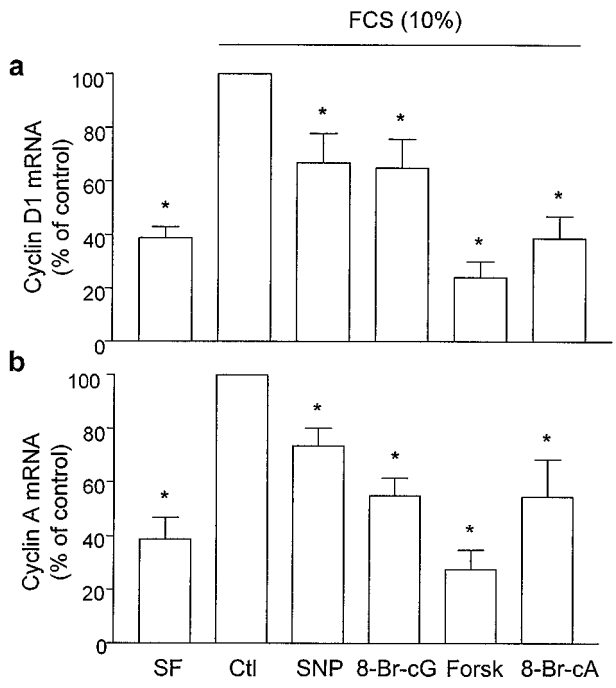

Figure 8 Cumulative data from Northern blots showing the effect of cyclic GMP- and cyclic AMP-dependent vasodilators on the FCS-induced (a) cyclin D1 and (b) cyclin A mRNA expression in cultured rat aortic smooth muscle cells. VSMC were exposed to FCS (10%) for 16 h. The vasodilators were added 5 min prior to FCS. Results shown as mean ± s.e.mean of at least four experiments. (*$P<0.05$ vs Ctl). Serum-free: SF; control: Ctl; SNP: 300 μM; 8-Br-cyclic GMP: 8-Br-cG, 3 mM; forskolin: Forsk, 10 μM; 8-Br-cyclic AMP: 8-Br-cA, 0.3 mM.

is involved. NO donors have also been reported to inhibit DNA synthesis in VSMC synchronized in the S phase, an effect which was not mimicked by 8-Br-cyclic GMP (Sarkar *et al*., 1997). Moreover, NO produced following induction of NOS II blocked macrophage-like cells in the early G2/M phase of the cell cycle (Takagi *et al*., 1994). Since experiments have not been performed with VSMC synchronized in the S phase, an inhibitory effect of the cyclic GMP- and cyclic AMP-dependent vasodilators on the progression of VSMC through the S and G2/M phases cannot be ruled out in the present study.

Thus, NO donors may arrest the cell cycle *via* at least two different mechanisms, a cyclic GMP-dependent arrest of VSMC up to the late G1, and a cyclic GMP-independent blockage of cells in the S phase. Such a cyclic GMP-independent blockade in the S phase may be due to an inhibition of ribonucleotide reductase or thymidine kinase (Garg & Hassid, 1993; Kwon *et al*., 1991).

In the present study, treatment of VSMC with SNP or 8-Br-cyclic GMP, in addition to preventing S phase entry, also impaired the expression of both G1 cyclin D1 and early S phase cyclin A mRNA and protein, whereas the expression of cyclin E was only minimally affected. Although a tight interegulation of the expression of cyclins has been demonstrated (Pines, 1995), the lack of effect on cyclin E expression might indicate that even low cyclin D1 levels may be sufficient to activate pathways leading to maximal cyclin E expression. The present findings are in agreement with those of a recent study reporting an impaired serum-stimulated expression of cyclin A but not of cyclin E by SNP and SNAP in rat aortic VSMC (Guo *et al*., 1998). In addition, in human umbilical artery VSMC, SNAP reduced the serum-stimulated expression of cyclin E and A mRNA, but not cyclin D1 mRNA, whereas no effect was found at the protein level (Ishida *et al*., 1997). The heterogenous effect of NO donors on the cyclin D1, E and A expression may be due to the use of different VSMC (i.e., species, arteries), but possibly also to the different ability of SNAP and SNP to release NO. Indeed, the antiproliferative effect of NO donors is critically dependent on the duration of NO release (Mooradian *et al*., 1995). Furthermore, experimental conditions associated with the continuous release of small amounts of NO (i.e. gene transfer of NOS III, cytokine-induced expression of NOS II, cocultures of endothelial cell and VSMC) strongly inhibited proliferation (Scott-Burden *et al*., 1992; Scott-Burden & Vanhoutte, 1993; Kullo *et al*., 1997), whereas the release of NO by a large bolus of NO donors during a short time-period was much less effective (Mooradian *et al*., 1995). In the present study a small but consistent inhibition of proliferation was observed by daily addition of SNP to VSMC, an effect that might have been further increased by a more frequent addition of the NO-donor. Beside the reduction of GP- and early S phase cyclin expression, the antiproliferative effect of NO donors may also involve an inhibition of cyclin-dependent kinase 2 or an enhanced expression of the cyclin-dependent kinase inhibitor p21 (Ishida *et al*., 1997).

In the current study, forskolin and 8-Br-cyclic AMP, analogous to the cyclic GMP-dependent vasodilators, inhibited the progression of VSMC from the G0/G1 phase into the S phase of the cell cycle, and reduced the expression of both cyclin D1 and A mRNA and protein, whereas the expression of cyclin E was only slightly inhibited. Consistent with these findings, cyclin D1 and A expression has been reported to be under negative control of cyclic AMP in different cell types, including fibroblasts (Desdouets *et al*., 1995; Barlat *et al*., 1995; L'Allemain *et al*., 1997), macrophages (Cocks *et al*., 1992) and an astrocytic cell line (Gagelin *et al*., 1994). The cyclic AMP-dependent vasodilators inhibited the FCS-stimulated expression of cyclin A to a greater extent than the cyclic GMP-dependent vasodilators. Thus, it is not unlikely that cyclic GMP acts *via* cross-activation of the cyclic AMP effector pathway. Such an assumption is supported by the findings that elevations of cyclic GMP levels in VSMC are associated with an activation of protein kinase A and that selective inhibitors of protein kinase A prevented the NO-mediated growth inhibition (Cornwell *et al*., 1994). Moreover, cyclic GMP has been shown to inhibit the degradation of cyclic AMP by phosphodiesterase III (Beavo & Reifsnyder, 1990).

Inhibitory effects of the cyclic GMP- and cyclic AMP-dependent vasodilators on the expression of cyclin D1 and A mRNA could reflect a repressed transcription of the cyclin D1 and A genes, respectively, and/or an increased degradation of the mRNA. Transcriptional regulation of the cyclin D1 and A genes could be mediated by the cyclic AMP responsive element, of which the consensus sequence has been identified in the promoters of these genes (Desdouets *et al*., 1995; Barlat *et al*., 1995; Herber *et al*., 1994). In contrast to the cyclin D1 and A genes, no cyclic AMP responsive element has been found in the promoter region of the cyclin E gene (Koff *et al*., 1991), a fact that could help explain the lack of effect of the cyclic GMP- and cyclic AMP-dependent vasodilators on cyclin E expression. Mutational analysis studies using promoter constructs of the cyclin A gene have shown that the cyclic AMP responsive element is required for the antiproliferative effect of cyclic AMP-elevating agents in fibroblasts (Barlat *et al*., 1995).

An impaired bioavailability of endothelium-derived NO seems to be one of the early events during the development of

atherosclerosis (Busse & Fleming, 1996). The nature of this endothelial dysfunction is largely unknown, although possible mechanisms include decreased substrate availability, decreased expression of NOS III, imbalance between endothelium-derived relaxing and constricting factors, production of an endogenous NOS inhibitor and overproduction of oxygen free radicals. In human atherosclerotic plaques and also in restenotic lesions of rat aortae after balloon injury, a marked induction of cell cycle regulatory proteins, such as cyclin A, cyclin E, and their associated kinase, cyclin-dependent kinase 2, and also proliferating cell nuclear antigen was found, predominantly in areas rich in VSMC (Wei *et al*., 1997). These previous findings in conjunction with the present data, support the concept that removal of the inhibitory feedback of NO on the expression of G1 and early S phase cyclins might contribute to the excessive VSMC proliferation leading to intimal thickening at sites of vascular injury.

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