Mechanisms mediating the antiproliferative effects of nitric oxide in cultured human airway smooth muscle cells

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Abstract We have characterised the mechanisms involved in the antiproliferative effect of NO in human airway smooth muscle cells (HASMC). *S*-Nitroso-*N*-acetyl penicillamine, a nitric oxide donor, inhibited proliferation in both G_1 and S phases of the cell cycle. Additionally, experiments with 8-bromo-cGMP, haemoglobin, a NO scavenger and zaprinast, a cGMP-specific phosphodiesterase inhibitor, showed that both effects were NO-mediated. The G_1 phase inhibition was cGMP-dependent whereas the S phase inhibition was due to a cGMP-independent inhibition of ribonucleotide reductase. These results demonstrate that NO inhibits HASMC proliferation by cGMP-dependent and -independent mechanisms acting at distinct points in the cell cycle.

Key words: Nitric oxide; cGMP; Smooth muscle; Remodelling; Asthma; Cell cycle

1. Introduction

In chronic asthma there is an increase in airway smooth muscle (ASM) mass, which appears to be due to hyperplasia and hypertrophy. This contributes to increased thickness of the airway wall and the resulting poorly reversible airflow obstruction, which characterises chronic asthma [1]. It is not clear whether the increase in ASM mass occurs as a result of overproduction of growth factors or loss of an inhibitory influence, or both. Recent studies have identified several growth factors for ASM in a number of species [2,3] but little is known about the mechanisms and pathways inhibiting ASM proliferation.

The synthesis of cGMP is catalysed by guanylyl cyclases (GC), which exist in two forms: a soluble (cytosolic) form, activated by nitric oxide (NO) [4], and a particulate (membrane-bound) form, activated by natriuretic peptides [5]. We have previously shown that both GC forms were abundant suggesting that they are functionally important [6]. NO is produced by the airway epithelium where it has a paracrine bronchoprotective role [7,8] and by non-adrenergic non-cholinergic nerves where it acts as an inhibitory neurotransmitter causing cGMP-mediated ASM relaxation [9,10]. NO has both cGMP-dependent and -independent effects on proliferation in other biological systems [11–14] and we have recently shown that continuous exposure to *S*-nitroso-*N*-acetyl penicillamine (SNAP) or 8-bromo-cGMP inhibits mitogen-induced proliferation in human airway smooth muscle cells (HASMC) [15]. HASMC had been shown to express type I nitric oxide synthase which results in inhibition of DNA synthesis and cell proliferation [16].

Regulation of cell cycle progression through the G_1 phase by extracellular factors is possible until the restriction point which occurs late in G_1 phase. This restriction point demarcates the commitment to subsequent DNA replication in the S phase and obligatory cell division in the M phase. Cells that have traversed the restriction point no longer require protein synthesis and are not dependent on extracellular growth factors. There is some evidence to suggest that the contractile properties of ASM are altered in asthma with promotion of the proliferative phenotype [17]. Endogenous antiproliferative substances such as transforming growth factor-β, heparin and prostaglandin E_2 [18,19] inhibit ASM cell progression at various points prior to the restriction point. This study characterises the intracellular mechanisms and cell cycle specificity of NO inhibition of HASMC proliferation. We used thymidine incorporation to measure proliferation in these studies but have previously shown that changes in thymidine incorporation are representative of changes in other proliferative indices such as cell counts or MTT incorporation after SNAP treatment [15].

2. Materials and methods

2.1. Materials

[^{3}H]Thymidine was obtained from Amersham Life Science (Little Chalfont, UK), foetal bovine serum (FBS) was purchased from JRH Biosciences (Sera-Lab, Sussex, UK). All other chemicals were purchased from Sigma-Aldrich (Poole, UK). Plasticware was purchased from Costar (Cambridge, MA, USA).

2.2. Cell culture

Primary cultures of HASMC were prepared from explants of ASM from two donors according to methods previously described [6,20,21]. We have shown, using morphological and immunohistochemical staining, that this method produces a relatively pure (>95%) population of ASM cells.

2.3. [^{3}H]Thymidine incorporation

Frozen aliquots of cells were thawed before use and plated at a density of 2×10^4 cells/ml in 96-well culture plates containing Dulbecco's modified Eagle's medium supplemented with 10% FBS, antibiotics and 4×10^{-3} M glutamine. At confluence, cells were growth-arrested by serum deprivation for 24 h. Experimental agents were added either at the time of the addition of FBS for 24 h (G_0/G_1 effect) or 24 h

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Abbreviations: HASMC, human airway smooth muscle cells; SNAP, *S*-nitroso-*N*-acetyl penicillamine; Hb, haemoglobin; MB, methylene blue; PDE, phosphodiesterase; FBS, foetal bovine serum

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later when cells re-entered the S phase for 1 h. 4 μCi/ml of [^{3}H]thymidine was added 24 h after addition of FBS (10 min after the delayed addition of drugs) for one additional hour. DNA was isolated using a cell harvester (Automash 2000, Dynatec, West Sussex, UK), adsorbed onto fibreglass filters, treated with 200 μl 0.01 M potassium hydroxide and immersed in 4 ml scintillation fluid (Ready protein, Beckman, Fullerton, CA, USA). Radioactivity was quantified using a scintillation counter (Minaxi 4000, Packard, CT, USA) with a counting efficiency of 40%.

2.4. Analyses

Results are shown as means ± S.E.M. [^{3}H]Thymidine incorporation data are expressed as dpm/well (or as % inhibition of stimulated controls to allow comparison between experimental protocols). The significance of drug effect was assessed by one-way analysis of variance followed by Student's *t*-test using the SPSS software program (SPSS, Chicago, IL, USA). A P value <0.05 was regarded as significant.

3. Results

3.1. Cell cycle synchronisation

Re-addition of serum-containing medium resulted in entry of cells into the S phase after 18 h, as determined by thymidine incorporation into DNA (Fig. 1). Serial FBS-induced thymidine incorporations after re-stimulation were (fold increase over unstimulated controls): 0.7 ± 0.1 at 6 h, 1.2 ± 0.06 at 9 h, 1.0 ± 0.2 at 12 h, 1.4 ± 0.3 at 15 h, 2.1 ± 0.1 at 18 h and 26 ± 1 at 24 h ($n=6$ for all, $P=0.27$, 0.15, 0.97, 0.16, <0.001 and <0.001 respectively).

According to these results, we consequently chose to measure thymidine incorporation in all experiments 24 h after addition of serum, and evaluated S phase effects of drugs during a 1 h window from 24 to 25 h after addition of serum.

3.2. Cell cycle studies

To determine the point of action of NO and cGMP in the cell cycle progression from G_0 through G_1 into the S phase, a delayed addition approach was used. At the indicated times (Fig. 2) after serum re-stimulation of growth-arrested cells, 10^{-4} M SNAP or 10^{-3} M 8-bromo-cGMP were added. When cells entered the S phase, 24 h after serum re-addition, DNA synthesis was measured for 1 h by thymidine incorporation. SNAP inhibited FBS-induced thymidine incorporation when it was added in the G_0/G_1 or the S phases. 10^{-4} M SNAP inhibited FBS-induced mitogenesis by 61 ± 3.8% at time 0, 68 ± 3.1% at 6 h, 60 ± 3.4% at 12 h and 68 ± 5.0% at 24 h ($n=6$, $P<0.001$ for all). In contrast, 8-bromo-cGMP inhibited FBS-induced thymidine incorporation only when added during the G_0/G_1 phase of the cell cycle. 10^{-3} M 8-bromo-cGMP inhibited FBS-induced mitogenesis by 39 ± 5.3% at time 0, 49 ± 4.4% at 6 h and 46 ± 4.2% at 12 h ($n=6$, $P<0.001$ for all), while it was without effect when added 24 h after serum addition ($n=6$, $P=0.43$).

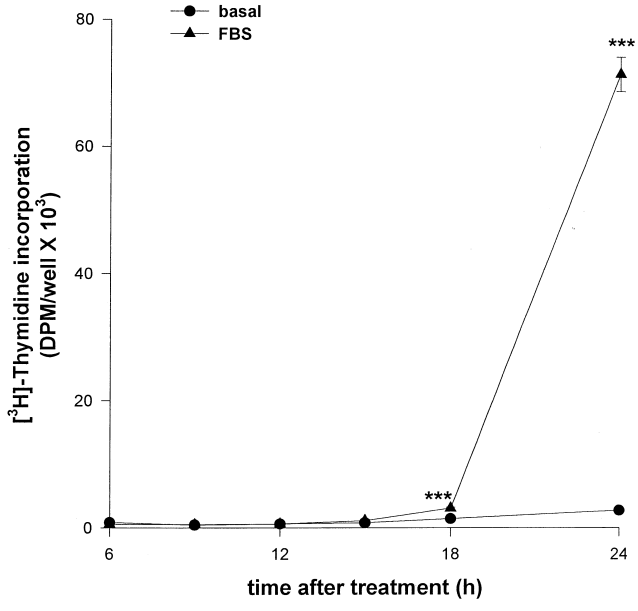

Fig. 1. Stimulation of DNA synthesis in HASMC by FBS. Growth-arrested cells were stimulated with 10% FBS for 6–24 h as described. Data represent the mean ± S.E.M., $n=6$. Significantly different from serum-free control: ***$P<0.001$.

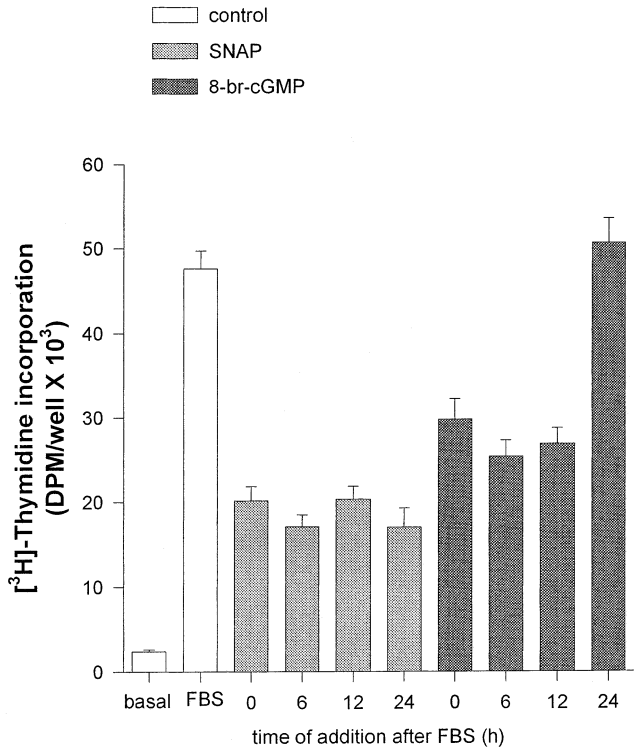

Fig. 2. Inhibition of FBS-induced DNA synthesis by SNAP and 8-bromo-cGMP. Growth-arrested cells were stimulated by 10% FBS for 24 h. 10^{-4} M SNAP and 10^{-3} M 8-bromo-cGMP were added at different time points after addition of FBS. Data represent the mean ± S.E.M., $n=6$.

3.2.1. The effect of haemoglobin on the antimitogenic effect of SNAP. To determine if the inhibitory effect of SNAP was related to NO donation we studied the effect of haemoglobin (Hb), an NO scavenger, on the antimitogenic effect of SNAP. Cells were pretreated with 10^{-5} M Hb for 1 h before the addition of 10^{-4} M SNAP. Hb alone had an inhibitory effect on basal and mitogen-stimulated thymidine incorporation when added in the G_0/G_1 phase which was reported previously in these cells and other cell types [15]. The antimitogenic effect of SNAP in both G_0/G_1 and S phases was blocked by Hb pretreatment (Fig. 3).

3.2.2. The effect of methylene blue on the antimitogenic effect of SNAP. To test whether cGMP was involved in the antimitogenic effects of SNAP we studied the effect of methylene blue (MB), an inhibitor of soluble GC. Cells were pretreated with 2×10^{-6} M MB for 1 h before the addition of

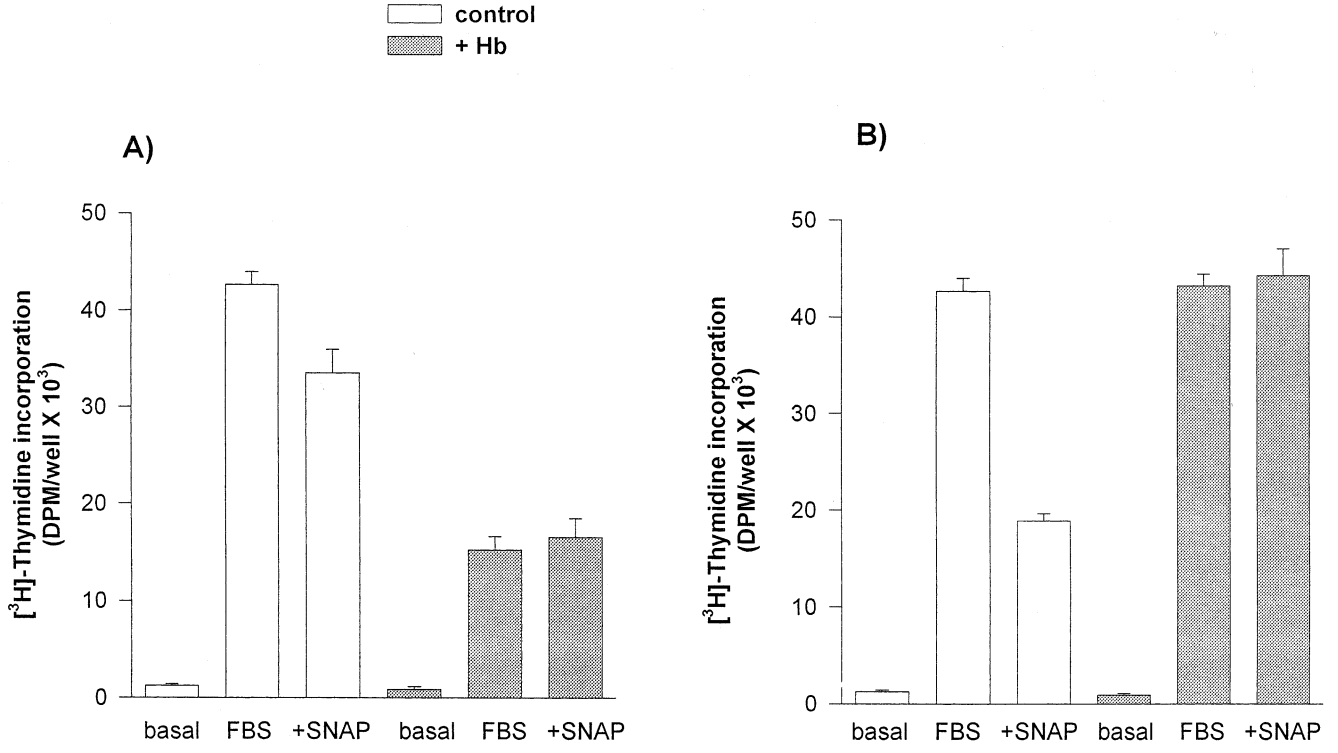

Fig. 3. Effect of Hb on the antimitogenic effects of SNAP added either during the G_0/G_1 phase (A) or in the S phase (B). Cells were pretreated with 10^{-5} M Hb for 1 h before the addition of 10^{-4} M SNAP. Data represent the mean ± S.E.M., $n = 6$.

10^{-4} M SNAP. MB alone had no significant effect on basal and mitogen-stimulated thymidine incorporation. The antimitogenic effect of SNAP in both G_0/G_1 and S phases was blocked by MB pretreatment (Fig. 4).

3.2.3. Effects of zaprinast on the antimitogenic effect of SNAP. To further test whether cGMP was involved in the antimitogenic effects of SNAP we studied the effect of zaprinast, a cGMP-specific phosphodiesterase (PDE) inhibitor [22].

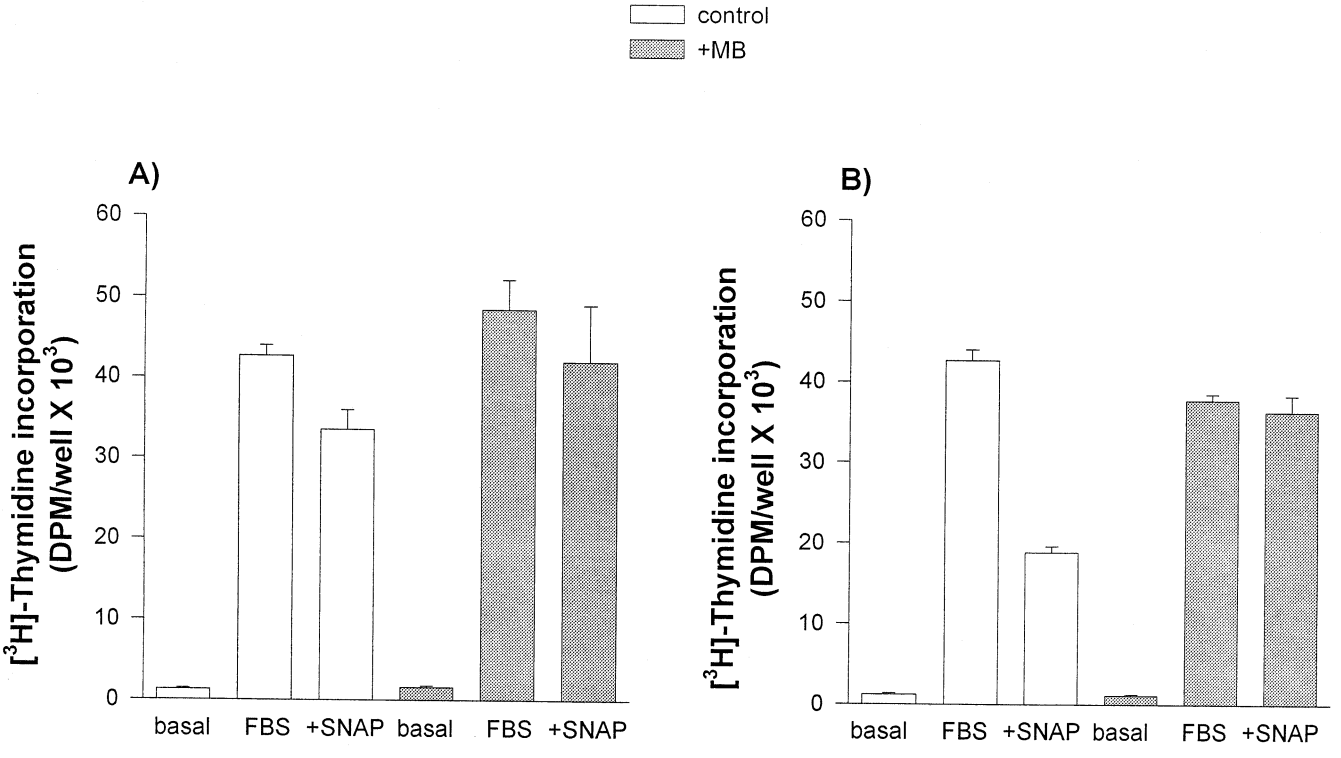

Fig. 4. Effect of MB on the antimitogenic effects of SNAP added either during the G_0/G_1 phase (A) or in the S phase (B). Cells were pretreated with 2×10^{-6} M MB for 1 h before the addition of 10^{-4} M SNAP. Data represent the mean ± S.E.M., $n = 6$.

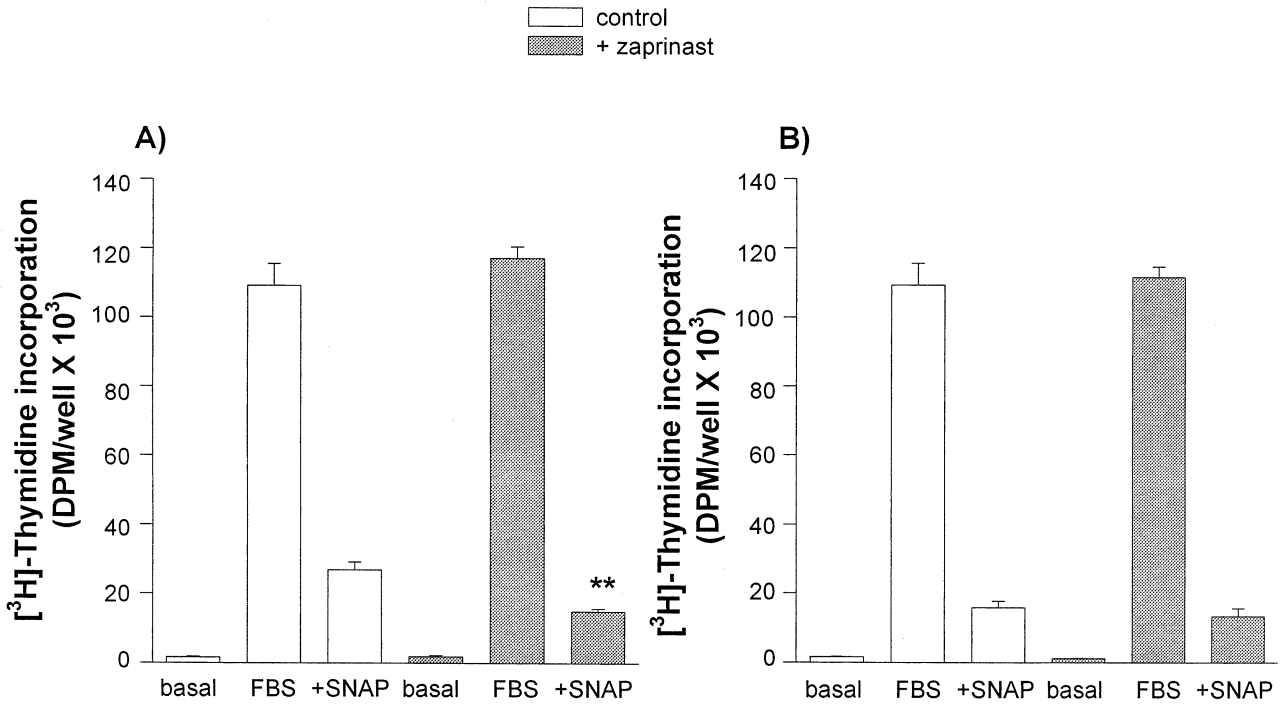

Fig. 5. Effect of zaprinast on the antimitogenic effects of SNAP added either during the G_0/G_1 phase (A) or in the S phase (B). Cells were pretreated with 10^{-6} M zaprinast for 1 h before the addition of 10^{-4} M SNAP. Data represent the mean ± S.E.M., $n = 6$. Significantly different from SNAP alone: $^{**}P < 0.01$.

Zaprinast alone did not alter basal or FBS-induced proliferation, however, the antimitogenic effect of SNAP, in G_0/G_1 but not S phase, was enhanced in the presence of zaprinast (Fig. 5).

3.2.4. The effect of deoxynucleosides on the antimitogenic effect of SNAP. To probe the mechanisms involved in the antimitogenic effects of SNAP in the S phase, we studied the effect of deoxynucleosides, to bypass ribonucleotide reductase.

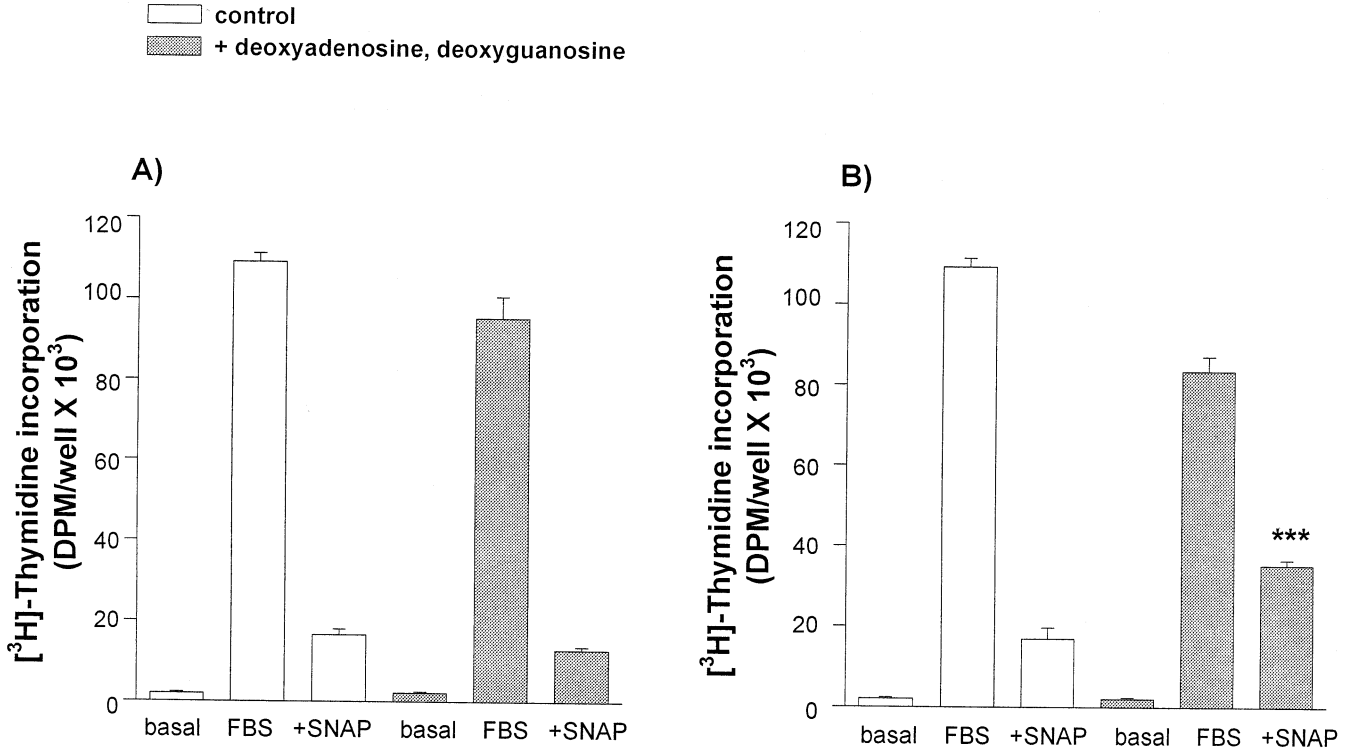

Fig. 6. Effect of deoxynucleosides on the antimitogenic effects of SNAP added either during the G_0/G_1 phase (A) or in the S phase (B). Cells were pretreated with 10^{-4} M deoxynucleosides for 1 h before the addition of 10^{-4} M SNAP. Data represent the mean ± S.E.M., $n = 6$. Significantly different from SNAP alone: $^{***}P < 0.001$.

Cells were pretreated with 10^{-4} M deoxynucleosides for 1 h before the addition of 10^{-4} M SNAP. The antimitogenic effect of SNAP in the S phase, but not G_0/G_1 phase, was partially reversed by deoxynucleoside pretreatment (Fig. 6).

3.3. Assessment of cell viability

None of the chemicals or vehicles used caused inhibition below the mean values for unstimulated cells when proliferation was assessed by thymidine incorporation, MTT assay or cell counting, nor was there evidence of cytotoxicity when measured by trypan blue exclusion.

4. Discussion

The major findings in this study are that NO inhibits HASMC proliferation by distinct mechanisms acting at different points in the cell cycle. cGMP-dependent effects were localised to late in the G_1 phase while NO inhibited DNA synthesis in the S phase via a cGMP-independent mechanism. This is the first report to characterise an endogenous substance inhibiting proliferation of ASM cells after the restriction point of cell cycle control in the late G_1 phase at which cell commit themselves to DNA synthesis in the S phase and subsequent cell division.

We have shown previously that SNAP elevates intracellular cGMP in cultured HASMC within minutes [6] and the antimitogenic effect of exogenous 8-bromo-cGMP suggests involvement of the cGMP pathway in the antiproliferative effects of NO in HASMC [15]. Our cell cycle approach identifies the point of action of the NO–cGMP mechanism and delineates antiproliferative actions of NO which are independent of cGMP. Our finding that cGMP and SNAP act late in the G_1 phase supports the concept that NO-induced cGMP acts via cross-activation of cAMP-dependent protein kinase [23], because inhibition of HASMC proliferation by cAMP has been localised to the late G_1 phase of the cell cycle [24].

The antiproliferative effect of SNAP in our study was likely to be NO-mediated as Hb, an NO scavenger which acts in a competitive manner [25], abolished the antimitogenic effect of SNAP. This is consistent with previous studies in other cell systems [26–28]. Zaprinast, a cGMP-specific PDE inhibitor, given at a concentration selective for cGMP-specific PDE [22], potentiated the antimitogenic effect of SNAP in the G_0/G_1 phase but not in the S phase of the cell cycle suggesting cGMP-dependent and -independent mechanisms respectively. The strongest evidence that the S phase inhibitory effects of SNAP are cGMP-independent is the loss of the inhibitory effect of exogenous 8-bromo-cGMP in the S phase and the preservation of the inhibition by SNAP when added in the S phase. These results contrast with the effect of MB, an inhibitor of soluble GC, which blocked the inhibition by SNAP in the G_0/G_1 and S phases. This discrepancy could be explained by the fact that MB can inactivate NO by generating superoxide radicals. In view of this limitation of MB, we tried a more selective inhibitor of soluble GC, 1*H*-[1,2,3]oxadiazolo-[4,3-*a*]quinoxalin-1-one, however it had a toxic effect over the time course of our experiments and could not be used. Exogenous deoxynucleosides reversed the antiproliferative effect of SNAP in the S phase, but not the G_0/G_1 phase of the cell cycle suggesting that ribonucleotide reductase inhibition is involved. However, the reversal by deoxynucleosides was partial indicating that other undefined mechanisms besides ribonucleotide reductase may be responsible for inhibition of DNA synthesis by NO. These results are consistent with previous data in vascular smooth muscle cells [11].

The ability of NO to inhibit the growth of HASMC in cells with altered G_1 control mechanisms may be significant to both the pathogenesis and the treatment of asthma. The importance of the restriction point as a cell cycle checkpoint was made evident by the finding that cancerous cells have diminished sensitivity at this point. Moreover, in cardiovascular diseases a number of vascular smooth muscle cell phenotypes have been found with decreased sensitivity to endogenous inhibitors that can act in the G_1 phase, e.g. transforming growth factor-β1. We speculate that the development of ASM phenotypes resistant to endogenous growth inhibitors that act in the late G_1 phase, coupled with decreased endogenous NO production due to epithelial shedding in asthma, which can inhibit the proliferation of ASM cells beyond the restriction point, may contribute to the increased ASM mass in asthma.

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