Antiproliferative effects of NO and ANP in cultured human airway smooth muscle

AHMED M. HAMAD, SIMON R. JOHNSON, AND ALAN J. KNOX
Division of Respiratory Medicine, City Hospital, Nottingham NG5 1PB, United Kingdom

Hamad, Ahmed M., Simon R. Johnson, and Alan J. Knox. Antiproliferative effects of nitric oxide and atrial natriuretic peptide in cultured human airway smooth muscle. Am. J. Physiol. 277 ( Lung Cell. Mol. Physiol. 21): L910–L918, 1999.—Airway smooth muscle (ASM) hyper trophy and hyperplasia are important determinants of bronchial responsiveness in asthma, and agents that interfere with these processes may prevent airway remodeling. We tested the hypothesis that activators of soluble and particulate guanylyl cyclases would inhibit human ASM cell (HASMC) proliferation. We report that the nitric oxide (NO) donors S-nitroso-N-acetylpenicillamine (SNAP; 10−6 to 10−4 M) and sodium nitroprusside (10−5 to 10−3 M) and human atrial natriuretic peptide [ANP-(1—28); 10−8 to 10−6 M], which activate soluble and particulate guanylyl cyclases, respectively, inhibited serum- and thrombin-induced proliferation of cultured HASMCs. The antimitogenic effect of SNAP was reversed by hemoglobin (10−5 M), an NO scavenger, suggesting that NO donation was involved. The antiproliferative effects of SNAP and ANP-(1—28) were potentiated by the cGMP-specific phosphodiesterase zaprinast and mimicked by 8-bromo-cGMP (10−3 M), suggesting that cGMP-dependent mechanisms were involved. However, first, ANP-(1—28) produced a smaller antiproliferative effect than SNAP in contrast to their abilities to elevate cGMP, and second, rat ANP-(104—126), which binds selectively to ANP clearance receptors without elevating cGMP, had a small antiproliferative effect, suggesting that cGMP-independent mechanisms were also involved. These results provide evidence for a novel antiproliferative effect of NO and ANP in HASMCs mediated through cGMP-dependent and cGMP-independent mechanisms.

nitric oxide; atrial natriuretic peptide; asthma; guanosine 3',5'-cyclic monophosphate; hypertrophy; hyperplasia

HISTOPATHOLOGICAL STUDIES of airways removed at postmortem from patients with severe asthma show that the area of airway smooth muscle (ASM) is substantially increased in both large (10, 24, 27, 58) and small (50) airways. This ASM thickening is believed to be one of the principal contributors to airway wall thickening and the resulting poorly reversible airflow obstruction, which characterizes chronic asthma (29). It is not clear whether the increase in ASM mass occurs as a result of overproduction of growth factors, a loss of inhibitory influence, or both. Recent studies (25, 32) have identified several growth factors for ASM in a number of species, but little is known about the mechanisms and pathways inhibiting ASM proliferation. Ligands binding to adenyl cyclase-linked receptors (β2-adrenoce-
We found that activators of soluble GC [S-nitroso-N-acetylpenicillamine (SNAP) and sodium nitroprusside (SNP)] and particulate GC [ANP-(1—28)] had potent inhibitory effects on serum- and thrombin-induced proliferation of HASMCs and that these effects occurred through cGMP-dependent and cGMP-independent pathways. Our studies suggest that activation of GCs may provide a novel target for the prevention of airway remodeling in asthma.

MATERIALS AND METHODS

Cell Culture

Primary cultures of HASMCs were prepared from explants of ASM according to methods previously described (21, 43, 44). We (21) have shown, using morphological and immunohistochemical staining, that this method produces a relatively pure (~95%) population of ASM cells. Frozen aliquots of cells were thawed before use and plated at a density of 2 × 10^4 cells/ml in culture plates containing DMEM plus 10% fetal bovine serum (FBS), 50 U/ml of penicillin G, 50 µg/ml of streptomycin, 2.5 µg/ml of amphotericin B, and 4 × 10⁻³ M l-glutamine. All experiments were performed in confluent HASMCs (unless otherwise stated) that had been growth arrested for 24 h by serum deprivation. Cells from five separate donors were used (passages 3–5).

Proliferation Assays

[³H]thymidine incorporation. Cells were grown in 96-well plates in DMEM plus 10% FBS until 90–100% confluent. The cells were then growth arrested in serum-free DMEM for 24 h. Growth-arrested cells were used because they can be synchronized in the G₀/G₁ phase of the cell cycle and at this baseline minimally incorporate [³H]thymidine (42). The cells were stimulated with either 10% FBS or 1 U/ml of human thrombin for 30 h. During the last 6 h, HASMCs were labeled with 4 µCi/ml of [³H]thymidine. DNA was isolated with a cell harvester (Automash 2000, Dynatec) adsorbed onto fiberglass filters, treated with 200 µl of 0.01 M potassium hydroxide, and immersed in 4 ml of scintillation fluid (Ready Protein, Beckman). Radioactivity was quantified with a scintillation counter (Minaxi 4000, Packard) with a counting efficiency of 40%.

Cell counts. Cells were grown in DMEM plus 10% FBS in 24-well plates for 2–3 days (until 30–40% confluent) and then growth arrested in serum-free DMEM for 24 h. They were then treated with either DMEM alone or DMEM plus 10% FBS with and without the appropriate test substance (day 0). Fresh medium containing the same concentration of the test substance was added every 48 h over 8 days. An MTT assay was performed every second day as previously described (45). Briefly, 20 µl of a 5 mg/ml MTT solution were added to each well, and the cells were incubated at 37°C for 1 h. After removal of the medium, 200 µl of DMSO were added to solubilize the blue formazan product, and the plates were shaken for 5 min. The optical density at 570 nm was compared with that of control wells with a plate reader (MR 5000, Dynatec).

Cyclic GMP Measurement

Cyclic GMP was measured as previously described (21). Briefly, the cells were trypsinized and cGMP was extracted with 0.1 M hydrochloric acid. The resulting suspension was freeze-dried (SB9, Lab Plant, Huddersfield, UK) before the measurement of cGMP content. cGMP was measured with a commercially available enzyme-linked immunosorbant assay kit (Amersham, Little Chalfont, UK). The samples were first acetylated with a mixture of acetic anhydride and triethylamine to increase the sensitivity of the assay to 4 fmol/100 µl. The coefficient of variation of the assay was 11%. All samples were assayed in duplicate. The cells from six wells not used for cGMP assay were used to give a representative estimate of protein content in each experiment with the method of Bradford (5).

Chemicals

[³H]thymidine was obtained from Amersham. FBS was purchased from JRH Biosciences (Sera-Lab). Human ANP-(1—28) was purchased from Novabiochem (Nottingham, UK). Rat ANP-(104—126) was purchased from Peninsula Laboratories Europe (Merseyside, UK). All other chemicals were purchased from Sigma-Aldrich (Poole, UK). plasticware was purchased from Costar (Cambridge, MA).

Analyses

Results are shown as means ± SE of the indicated number (n) of individual observations from representative experiments. Each experiment was repeated two to three times with similar results. [³H]thymidine incorporation data are expressed as disintegrations per minute per well (or as percent inhibition of stimulated control cells to allow comparison between experimental protocols). MTT assay data are expressed as absorbance at 570 nm per well. Cell-counting data are expressed as cell number per well. Significance of the drug effect was assessed by one-way ANOVA followed by Student’s t-test with the SPSS software program (SPSS, Chicago, IL). A P value ≤0.05 was regarded as significant.

RESULTS

Effect of Serum and Thrombin on HASMC Proliferation

Preliminary experiments showed that a 30-h time point was optimal for measuring thymidine incorporation in cells stimulated with FBS or thrombin (data not shown). Both FBS and thrombin caused a concentration-dependent increase in thymidine incorporation, with a maximal increase of 56 ± 4.2- and 8 ± 0.4-fold in cells treated with 10% FBS and 1 U/ml of thrombin, respectively (P < 0.001 for both; n = 6; Fig. 1).
Effect of SNAP on HASMC Proliferation

SNAP concentration dependently inhibited thymidine incorporation induced by 10% FBS and 1 U/ml of thrombin. FBS-induced thymidine incorporation was inhibited by 31 ± 7.0% at 10⁻⁶ M, 52 ± 3.2% at 10⁻⁵ M, and 62 ± 6.1% at 10⁻⁴ M SNAP (P < 0.05, 0.001, and 0.001, respectively; n = 6; Fig. 2A). Thrombin-induced thymidine incorporation was inhibited by 73 ± 14.6% at 10⁻⁵ M SNAP and was abolished at 10⁻⁴ M SNAP (P < 0.01 and 0.001, respectively; n = 6; Fig. 2B). Similar results were obtained with SNP (Fig. 3).

Because thymidine incorporation measures DNA synthesis that may occur in some systems without cell division (8), we compared data from thymidine incorporation experiments with data from MTT assays (26) and cell-counting experiments (with 10% FBS as the mitogenic stimulus). SNAP at 10⁻⁴ M abolished the FBS-induced increase in MTT conversion on day 4 and inhibited the FBS-induced increase in MTT by 78 ± 1.9 and 61 ± 4.6% on days 6 and 8, respectively (P < 0.001 for all; n = 12; Fig. 4A). In cell-counting experiments, 10⁻⁴ M SNAP inhibited the FBS-induced increase in cell number by 82 ± 12.7, 87 ± 2.7, 88 ± 1.6, and 91 ± 0.6% on days 2, 4, 6, and 8, respectively (P < 0.01, 0.001, 0.001, and 0.001, respectively; n = 4; Fig. 4B).

To determine whether the inhibitory effect of SNAP was related to NO donation, we studied the effect of hemoglobin (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 or with Hb alone. Although Hb alone had an inhibitory effect on basal and mitogen-stimulated thymidine incorporation, the antimitogenic effect of SNAP was blocked by Hb pretreatment. SNAP at 10⁻⁴ M inhibited FBS-induced thymidine incorporation by 88 ± 1.5 and 6 ± 0.4% in the absence and presence, respectively, of Hb (P < 0.001; n = 6; Fig. 5A). Similarly, 10⁻⁴ M SNAP inhibited thrombin-induced thymidine incorporation by 63 ± 7.5% in the absence of Hb, whereas it had no effect in the presence of Hb (P < 0.05; n = 6; Fig. 5B).

Effect of Human ANP-(1—28) on HASMC Proliferation

ANP-(1—28) concentration dependently inhibited thymidine incorporation induced by 10% FBS and 1 U/ml of thrombin. FBS-induced thymidine incorporation was inhibited by 21 ± 3.1% at 10⁻⁸ M, 45 ± 6.6% at 10⁻⁷ M, and 50 ± 3.4% at 10⁻⁶ M ANP-(1—28) (P = 0.1, P <
Thrombin-induced thymidine incorporation was inhibited by 21.6 ± 5.4% at 10^{-8} M, 31 ± 10.0% at 10^{-7} M, and by 33 ± 6.8% at 10^{-6} M ANP-(1—28) (P < 0.05, 0.05, and 0.01, respectively; n = 6; Fig. 6A).

Effect of Rat ANP-(104—126) on HASMC Proliferation

To determine whether ANP could have cGMP-independent antimitogenic effects, we studied the effect of ANP-(104—126), an ANP analog that binds selectively to ANP clearance receptors without elevating cGMP levels on FBS- and thrombin-induced proliferation. Rat ANP-(104—126) concentration dependently inhibited 10% FBS- and 1 U/ml of thrombin-induced thymidine incorporation, although to a lesser extent than human ANP-(1—28). FBS-induced thymidine incorporation was inhibited by 14 ± 4.1% at 10^{-6} M and 22 ± 3.4% at 10^{-5} M (P < 0.05 and 0.01, respectively; n = 6; Fig. 7A). Thrombin-induced thymidine incorporation was inhibited by 20 ± 7.8% at 10^{-7} M, 21 ± 6.5% at 10^{-6} M, and 25 ± 5.3% at 10^{-5} M (P = 0.1, P = 0.06, and P < 0.05, respectively; n = 6; Fig. 7B). We confirmed that rat ANP-(104—126) had no effect on cGMP levels in these cells (data not shown).

Effect of 8-Bromo-cGMP on HASMC Proliferation

To further elucidate the role of cGMP in the antimitogenic effect of SNAP and ANP-(1—28), we determined whether this effect could be mimicked by 8-bromo-cGMP (stable cell-permeable cGMP analog). 8-Bromo-cGMP concentration dependently inhibited 10% FBS- and 1 U/ml of thrombin-induced thymidine incorporation. FBS-induced thymidine incorporation was inhib-
Fig. 5. Effect of hemoglobin (Hb) on antimitogenic effects of SNAP in FBS (A-) and thrombin (B)-stimulated cells. Growth-arrested cells were pretreated with 10⁻⁵ M Hb for 1 h before stimulation with 10% FBS or 1 U/ml of thrombin in presence (+) of 10⁻⁴ M SNAP for 30 h. Data are means ± SE; n = 6 observations from representative experiments. Significantly different from SNAP alone: *P < 0.05; ***P < 0.001.

Fig. 6. Concentration-dependent inhibition of FBS (A-) and thrombin (B)-induced DNA synthesis by human atrial natriuretic peptide [ANP-(1—28)]. Growth-arrested cells were stimulated with 10% FBS or 1 U/ml of thrombin in presence of indicated concentrations of ANP-(1—28) for 30 h as described in METHODS. Data are means ± SE; n = 6 observations from representative experiments. Significantly different from control (mitogen-alone) cells: *P < 0.05; **P < 0.01; ***P < 0.001.
suggests that the effects were mediated, at least in part, by cGMP.

We used serum as a mitogenic stimulus because the increase in ASM growth seen with increasing concentrations of serum in culture may provide an in vitro correlate of the situation in vivo created by increased vascular permeability with plasma leakage in asthmatic airways (55). Thrombin was used as a more specific mitogen because it is generated at the sites of inflammation in the airways and has been shown to potently stimulate DNA synthesis in HASMCs (41). The change in thymidine incorporation produced by 10% FBS was significantly greater than that produced by 1 U/ml of thrombin, which is consistent with the results of previous studies in HASMCs (23, 57). The magnitude of the changes in the proliferative indexes seen in response to serum and thrombin in our study are broadly similar to those previously reported in HASMCs (41).

We used thymidine uptake as our main proliferation index. In selected experiments, we confirmed that the changes in thymidine incorporation were paralleled by the changes in MTT conversion and cell number, suggesting that thymidine uptake was a measure of cell proliferation under our experimental conditions.

We used SNAP as the main NO donor because it is a direct NO donor and produced the largest change in cGMP of NO donors in HASMCs in the previous study by Hamad et al. (21). Both FBS and thrombin induced significant increases in thymidine incorporation, which were prevented by SNAP and SNP. Similarly, the FBS-induced increase in cell number was inhibited by SNAP. SNAP and SNP inhibited thrombin-induced proliferation to a much greater extent than serum-induced proliferation, which may be due to the lesser degree of proliferation induced by thrombin (57). Alternatively, it may reflect differential inhibition of the intracellular pathways mediating the thrombin response. The inhibitory effects of NO donors may therefore relate to the type of mitogenic stimulus. The inhibition by SNAP of cell counts was greater than that by thymidine incorporation, consistent with experiments in vascular smooth muscle cells (17). This difference is likely to reflect the fact that these indexes measure different aspects of proliferation at different times related to cell cycling. Thymidine incorporation is a measure of thymidine uptake across the cell membrane as well as thymidine incorporation into DNA, whereas cell counts reflect the balance between cell proliferation and apoptosis.

The antiproliferative effect of SNAP in our study was likely to be NO mediated because Hb, an NO scavenger that acts in a competitive manner (18), reduced the antimitogenic effect of SNAP. This is consistent with previous studies in other cell systems (9, 13, 15). Hb had an antiproliferative effect on its own, which was reported in other cell systems (14, 15). This effect of Hb was not due to toxicity because we saw no effect of Hb on MTT assay or trypan blue exclusion (data not shown). Because Hb was dissolved in DMEM, there was no possibility of a vehicle effect. The mechanism, therefore, is not clear, but possibilities include enhanced release of antiproliferative cytokines in the presence of Hb or an oxygen-scavenging effect altering mitochondrial respiration. Having demonstrated an antimitogenic effect of SNAP, we performed experiments to probe the role of cGMP. Zaprinast, a cGMP-specific PDE inhibitor, given at a concentration selective for cGMP-specific PDE (3) potentiated the antimitogenic effect of SNAP parallel to an increase in cGMP accumulation. Moreover, the cell-permeable cGMP analog 8-bromo-cGMP could mimic the antimitogenic effect of SNAP, although it was less potent. Taken together, these results support the view

Table 1. Effects of zaprinast on actions of SNAP and ANP-(1—28) in HASMCs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>cGMP, pmol/mg protein</th>
<th>Thymidine Incorporation, % inhibition of response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FBS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thrombin</td>
</tr>
<tr>
<td>$10^{-4}$ M SNAP</td>
<td>4.6 ± 0.2</td>
<td>73±4.5</td>
</tr>
<tr>
<td>SNAP + zaprinast</td>
<td>9.9 ± 0.5</td>
<td>85±3.1</td>
</tr>
<tr>
<td>$10^{-6}$ M ANP</td>
<td>9.8 ± 0.5</td>
<td>47±2.6</td>
</tr>
<tr>
<td>ANP + zaprinast</td>
<td>13.7 ± 1.4</td>
<td>71±3.5</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 observations from representative experiments. SNAP, S-nitroso-N penicillamine; ANP, atrial natriuretic peptide; HASMC, human airway smooth muscle cell; FBS, fetal bovine serum. Significantly different from agonist alone: *P < 0.05; †P < 0.01; ‡P < 0.001.
that at least part of the effect of SNAP was mediated by cGMP. However, the fact that 8-bromo-cGMP was less effective than SNAP and SNP at inhibiting proliferation suggests that additional cGMP-independent mechanisms may also be involved. The suggestion that NO donors may have both cGMP-dependent and -independent effects has been made by workers in a variety of cell lines, including vascular smooth muscle cells (16, 37, 54). Several NO donors and the cGMP analog 8-bromo-cGMP have been shown to inhibit serum-induced mitogenesis and proliferation in cultured rat aortic and renal arteriolar smooth muscle cells via a cGMP-dependent mechanism (9, 15, 39). In contrast, NO inhibition of rat aortic smooth muscle cells is independent of cGMP (52, 53). Several cGMP-independent mechanisms have been suggested for the antiproliferative effect of NO, including inhibition of thymidine kinase (17) and ribonucleotide reductase, the rate-limiting enzyme in DNA synthesis (34). Future studies are needed to determine the mechanisms involved in HASMCs.

Further evidence for dissociation between the effect of GC activators on cGMP formation and proliferation comes from our studies with ANP. We used human ANP-(1—28) as an activator of particulate GC because Hamad et al. (21) previously showed it to be the most potent natriuretic peptide at elevating cGMP in HASMCS. Although we found that human ANP-(1—28), like SNAP, had antiproliferative effects, it produced a smaller maximum effect. This difference in efficacy contrasts with relative abilities to elevate cGMP (ANP > SNAP) (21). This paradox suggests that either additional cGMP-independent mechanisms are involved with SNAP or compartmentalization of cGMP pools enables cGMP generated by soluble GC to have a greater antiproliferative effect than that generated by particulate GC. The potentiation of the antimitogenic effect of human ANP-(1—28) by zaprinast suggests that additional cGMP-independent mechanism may contribute to the antiproliferative effects of ANPs. We confirmed that rat ANP-(104—126) had no effect on cGMP levels in our cells. These results are consistent with previous reports in rat aortic smooth muscle cells (6, 7) showing that ANP can exert antiproliferative effects in a cGMP-independent manner through the clearance receptors. It is also interesting that, in contrast to NO donors and 8-bromo-cGMP, ANP inhibited serum-induced proliferation to a greater extent than thrombin-induced proliferation, providing further evidence that the mechanisms involved in their antiproliferative effects are different.

The effects of SNAP and SNP in our studies were greater than those of ANP or 8-bromo-cGMP, which were only partially effective at inhibiting thymidine incorporation. The magnitude of these partial effects is comparable to the effects reported with other antiproliferative agents in ASM cells such as β2-adrenoceptor agonists and cAMP mimetics, corticosteroids, interleukin-4, and heparin (23, 30, 40, 57, 60).

Our results aid our understanding of the hyperplastic changes occurring in ASM in asthma. Disruption and loss of the bronchial epithelium is a well-described histological feature of asthma (32). Because this epithelium is an important source of NO, its loss in asthma could therefore lead to the removal of an important inhibitory paracrine mechanism and contribute to the increase in ASM bulk, which characterizes this condition. Epithelial loss would also deplete other antmitogenic protective factors such as PGE2 (12). We have also considered whether the antiproliferative effect of ANP might have a physiological role. The lung is capable of synthesizing ANP (47), and both types of ANP receptors have been characterized and localized throughout the lung. The specific binding sites per gram of tissue
weight or per whole organ in rats were found to be greater in the lung than in other tissues including the kidney (19). In the heart, where more is known about the functions of ANP, ANP release is stretch stimulated (11). Similarly, Springall et al. (56) suggested that stretch of rat pulmonary veins stimulates ANP release. Preliminary data support a similar stretch-dependent mechanism for ANP release in tracheal muscle because sustained stretch led to increased cGMP, but not cAMP, levels in the trachealis muscle of anesthetized sheep (46). Stretch-dependent release of ANP from ASM, occurring with deep inspiration, could lead to cGMP elevation, with subsequent inhibition of ASM tone and cell proliferation.

In conclusion, we have shown for the first time that NO donors and ANP can inhibit HASMC proliferation in culture. The fact that these effects were mimicked by a cell-permeable cGMP analog and potentiated by a cGMP-specific PDE inhibitor suggests that the effects are partly related to an elevation in cGMP. However, the fact that the relative potencies of SNAP and ANP do not reflect their ability to generate cGMP suggests that additional cGMP-independent effects are involved. The effects of NO and ANP may be important in developing strategies for the prevention of airway remodeling in chronic asthma.

A. M. Hamad was supported by the Egyptian Ministry of Higher Education.

Address for reprint requests and other correspondence A. J. Knox, Division of Respiratory Medicine, Clinical Science Bldg., City Hospital, Hucknall Rd., Nottingham NG5 1PB, UK (E-mail: Alan.knox@nottingham.ac.uk).

Received 16 December 1998; accepted in final form 1 July 1999.

REFERENCES


ROLE OF cGMP IN AIRWAY SMOOTH MUSCLE PROLIFERATION


