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A redox-based mechanism for nitric oxide-induced inhibition of DNA synthesis in human vascular smooth muscle cells

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1 The current study explored potential redox mechanisms of nitric oxide (NO)-induced inhibition of DNA synthesis in cultured human and rat aortic smooth muscle cells.

2 Exposure to S-nitrosothiols, DETA-NONOate and NO itself inhibited ongoing DNA synthesis and S phase progression in a concentration-dependent manner, as measured by thymidine incorporation and flow cytometry. Inhibition by NO donors occurred by release of NO, as detected by chemiluminescence and judged by the effects of NO scavengers, haemoglobin and cPTIO.

3 Co-incubation with redox compounds, N-acetyl-L-cysteine, glutathione and L-ascorbic acid prevented NO inhibition of DNA synthesis. These observations suggest that redox agents may alternatively attenuate NO bioactivity extracellularly, interfere with intracellular actions of NO on the DNA synthesis machinery or restore DNA synthesis after established inhibition by NO.

4 Recovery of DNA synthesis after inhibition by NO was similar with and without redox agents suggesting that augmented restoration of DNA synthesis is an unlikely mechanism to explain redox regulation.

5 Study of extracellular interactions revealed that all redox agents potentiated S-nitrosothiol decomposition and NO release.

6 Examination of intracellular NO bioactivity showed that as opposed to attenuation of NO inhibition of DNA synthesis by redox agents, there was no inhibition (potentiation in the presence of ascorbic acid) of soluble guanylate cyclase (sGC) activation judged by cyclic GMP accumulation in rat cells.

7 These data provide evidence that NO-induced inhibition of ongoing DNA synthesis is sensitive to redox environment. Redox processes might protect the DNA synthesis machinery from inhibition by NO, in the setting of augmented liberation of biologically active NO from NO donors.

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Abbreviations: ASC, L-ascorbic acid; cGMP, cyclic guanosine monophosphate; cPTIO, 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazonline-1-oxyl-3; DETA-NONOate, (Z)-1-[2-aminoethyl]-N-(2-ammonioethyl)amino]diazen-1-ium 1,2-diolate; DMEM, Dulbecco's modified Eagle's medium; FBS, Foetal bovine serum; GSH, glutathione; GSNO, S-nitrosoglutathione; IBMX, 3-isobutyl-1-methylxanthine; NAC, N-acetyl-L-cysteine; NAS, N-acetyl-L-serine; sGC, soluble guanylate cyclase; SMC, smooth muscle cells

Introduction

Excessive cellular proliferation is a characteristic feature of many human diseases, including tumour progression and vascular proliferative diseases such as atherosclerosis, restenosis after surgery and accelerated vasculopathy after heart transplantation (Ross, 1993). The highly reactive gaseous molecule nitric oxide (NO) is cytostatic to tumour cells, an action which involves inhibition of DNA synthesis (Kwon *et al*., 1991; Lepoivre *et al*., 1991). NO has also been shown to inhibit vascular smooth muscle cell proliferation associated with the formation of arterial lesions after balloon angioplasty and hypercholesterolaemia *in vivo*, (Cooke *et al*., 1992; McNamara *et al*., 1993) and in cultured cells *in vitro* (Garg & Hassid, 1993). NO exerts this antiproliferative effect at least partly by inhibiting DNA synthesis (Sarkar *et al*., 1997a,b). Although the enzyme, ribonucleotide reductase has been suggested as a target of NO (Kwon *et al*., 1991; Lepoivre *et al*., 1991; Sarkar *et al*., 1997a), cellular mechanisms regulating this response remain to be elucidated.

Intracellular redox status is being increasingly recognized to regulate the activity of a variety of processes including signal transduction, enzyme activity and metabolic functions as well as some responses brought about by exposure to NO (Sen & Packer, 1996; Stamler, 1994). Specifically, cellular sulphydrils appear to play a pivotal role in modulating NO action (Stamler *et al*., 1992). Reduced thiols might increase the half-life of NO by preventing its inactivation by oxygen free radicals. Furthermore, thiols appear to be necessary for the activity of NO synthases and guanylate cyclase in the production of cyclic GMP and mediating vasorelaxation (Ignarro & Gruetter, 1980; Pollock *et al*., 1991). NO has also been reported to react with endogenous thiols to form nitrosothiols and to modulate macromolecule function by covalent modification (Stamler, 1994). However, the role of redox processes and particularly the role of sulphydrils in NO-induced inhibition of DNA synthesis remains unknown. The objective of this study was to clarify some of these issues in cultured human aortic smooth muscles (SMC).

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Methods

Reagents

Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), trypsin-EDTA, glutamine, penicillin-streptomycin, phosphate buffered saline (PBS), hydroxyurea (HU), N-acetyl-L-cysteine (NAC), N-acetyl-L-serine (NAS), L-ascorbic acid (ASC), 8-Bromoguanosine 3′:5′-cyclic monophosphate (8-bromo-cyclic GMP), 3-isobutyl-1-methylxanthine (IBMX), 2′-deoxyadenosine and 2′-deoxyguanosine, antibodies for smooth muscle α-actin, HHF35 human actin and myosin were purchased from Sigma chemicals. Radiolabelled [^{3}H]-thymidine was from ICN and ^{125}I-labelled cyclic GMP was obtained from Amersham Life Sciences. Rabbit anti-cyclic GMP was purchased from Calbiochem. S-nitroso-L-glutathione (GSNO), S-nitroso-N-acetylpenicillamine (SNAP), (Z)-1-[2-aminoethyl] -N-(2-ammonioethyl)amino]diazen-1-ium 1,2-diolate (DETA-NONOate), 1H - [1,2,4]oxidiazole[4,3 - a]quinoxalin - 1 - one] (ODQ) and carboxy2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (cPTIO) were obtained from Alexis Biochemicals.

Cell culture

Human aortic SMC were cultured by the explant method from aortas of human donor heart allografts. Rat aortic SMC were kindly provided by Professor John D. Catravas from Vascular Biology Centre, Medical College of Georgia, Augusta, GA, U.S.A. The cells were identified by their typical hill-and-valley morphology in culture and their characteristic immunocytochemical staining for smooth muscle α-actin, HHF 35 human muscle actin and myosin. Cells were grown in Dulbecco's modified eagle medium (DMEM), supplemented with 2 mM glutamine, penicillin-streptomycin (100 unit ml^{-1}; 100 μg ml^{-1}, respectively) and 10% foetal bovine serum (FBS) at 37°C in a humidified incubator with 5% CO_2. Experiments were performed on human aortic SMC derived from three different donors and at passages lower than ten.

Cell synchronization, DNA synthesis and S phase progression

To investigate the influence of experimental agents on DNA synthesis, cells were initially synchronized by hydroxyurea to cause cell cycle arrest at the G1/S boundary. Synchronized cells were used as opposed to cycling cells to eliminate any influence that NO might exhibit on other stages of the cell cycle, such as early G1 signalling and G1 phase progression (Sarkar *et al*., 1997a). Cells were then allowed to restore DNA synthesis activity by removal of hydroxyurea and to progress through S phase in a synchronized manner 3–4 h after removal of hydroxyurea. The influence of experimental agents such as NO donors and redox agents on DNA synthesis and S phase progression was evaluated by thymidine uptake and flow cytometric analysis, respectively in this time frame and experimental setting. Incorporation of [^{3}H]-thymidine into acid insoluble macromolecules was determined after pulse labelling (1 μCi ml^{-1}) followed by extracting the cells with 10% TCA at 4°C for 30 min. Acid insoluble macromolecules were solubilized in 0.1% SDS/0.3 mol l^{-1} NaOH for 1 h. Radioactivity was measured by scintillation counting. For flow cytometry, synchronized cells at G1/S boundary were incubated in the absence and presence of experimental agents for 4 h. They were then harvested by trypsinization, fixed and permeabilized with absolute ethanol at 4°C for 10 min followed by treatment with 400 μg ml^{-1} RNase A and labelling with 600 μg ml^{-1} propidium iodide for 30 min at 37°C. Propidium iodide binding and determination of DNA content was analysed by a Coulter Epics XL flow cytometer.

NO analysis by chemiluminescence

Ongoing release of NO from S-nitrosothiols was monitored by the chemiluminescence principle, using a NO analyser (Sievers, Model 270B). Conditions were set to detect NO gas itself as opposed to biologically inactive stable degradation products of NO, such as, nitrite and nitrate. A continuous stream of N_2 gas was bubbled through 5 ml of serum-free tissue culture medium in the purge vessel in order to deliver any released NO gas to the analyser. Under these conditions, injection of NO donors (1–1000 μM) or NO gas itself, resulted in a positive NO signal. Furthermore, injection of sodium nitrite, up to 10 mM into the purge vessel did not result in a NO signal, but a NO signal was readily detectable under acidic conditions.

Measurement of cyclic GMP

Cultured human and rat aortic SMC or freshly obtained native human aorta pieces denuded of endothelium and cleaned from adventitia were preincubated for 10 min with the phosphodiesterase inhibitor, IBMX (1 mM) in order to prevent degradation of cyclic GMP. Tissue was then further treated with increasing concentrations of NO donors in the presence or absence of IBMX for 15 min. Cyclic GMP was extracted with 0.1 M HCl for 1 h and cyclic GMP content was quantified by standard radioimmunoassay according to Brooker *et al*. (1979). The assay was performed by acetylating the samples and standards with 2 : 1 mixture of triethylamine : acetic anhydride, by using a rabbit polyclonal antibody to cyclic GMP and charcoal to separate the antibody bound and unbound radioactive cyclic GMP.

Data analysis

Data are summarized by group and expressed as means ± standard error of mean (s.e.mean) of the indicated sample size or displayed as representative observations of at least three separate experiments. Statistical comparisons among groups were performed using ANOVA and Neuman-Kuels *post hoc* tests. Statistical significance was accepted at $P<0.05$.

Results

The DNA synthetic rate in human aortic SMC synchronized at G1/S phase boundary by treatment with 0.6 mM hydroxyurea for 12 h was negligible compared to the control, untreated cycling cells (419 ± 44 vs 18981 ± 3672 c.p.m. thymidine incorporation). DNA content, measured by flow cytometry also showed synchronization of hydroxyurea-treated cells in G1/S (Figure 1A). Removal of hydroxyurea and re-addition of 5% serum, resulted in increased DNA synthetic rate over 4 h, as shown by an increase in thymidine incorporation reaching a maximum 4–5 h after washout of hydroxyurea (4030 ± 1056, 28114 ± 1911 c.p.m. for 1 and 4 h, respectively). Flow cytometry also showed a synchronized progression of cells through S phase resulting in accumulation of cells in G2 phase after 4 h (Figure 1B).

Incubation of cells with S-nitroso-glutathione (GSNO) produced a concentration-dependent inhibition of DNA synthesis, as shown by thymidine incorporation (89.3 ± 1.5–

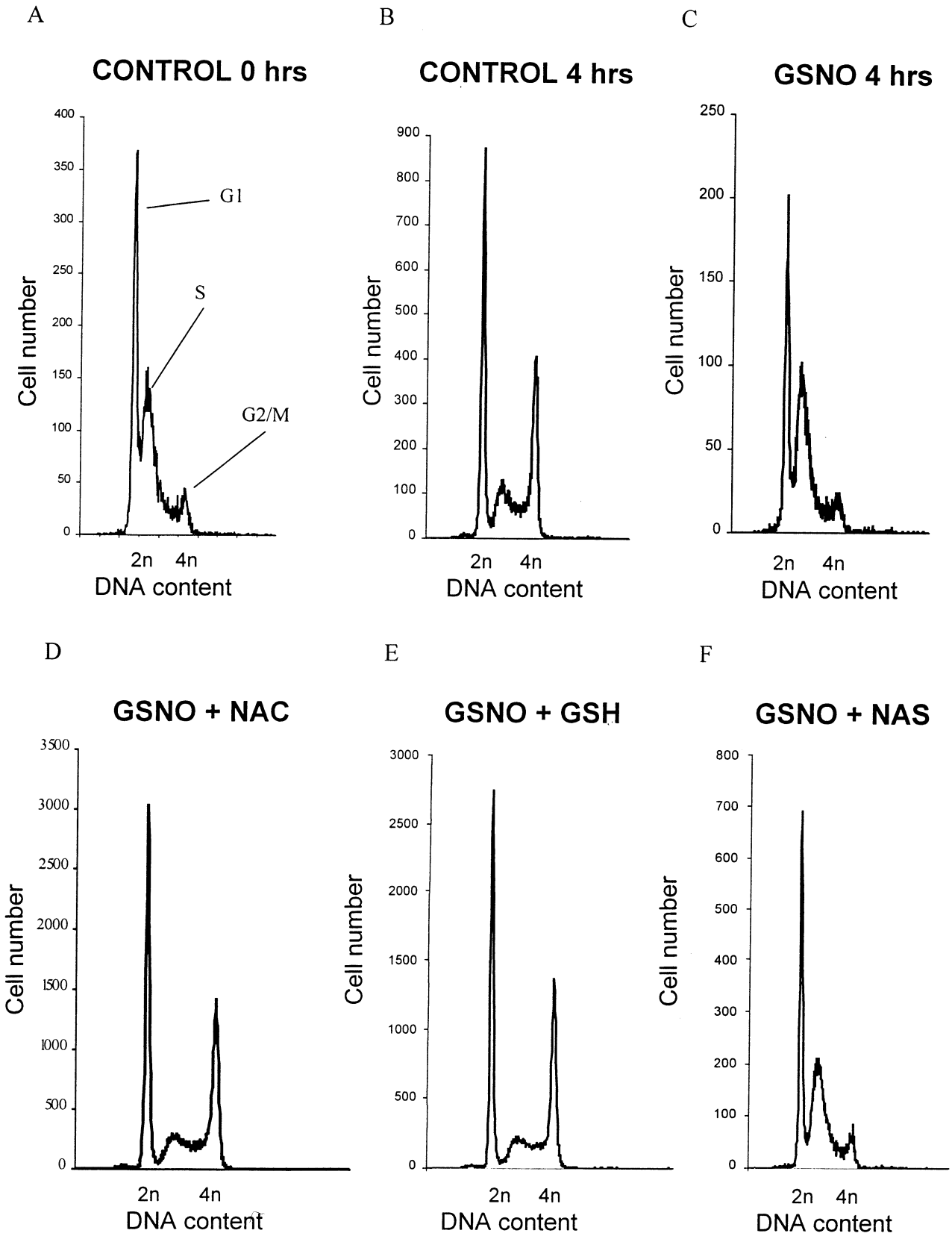

Figure 1 Influence of S-nitrosoglutathione (GSNO) on S phase progression after washout of hydroxyurea in human aortic smooth muscle cells (A–C) and the effects of N-acetylcysteine (NAC), glutathione (GSH) and N-acetylserine (NAS) on GSNO-induced inhibition of DNA synthesis (D–F). DNA content was monitored by flow cytometry in cells synchronized at G1/S boundary by 12 h hydroxyurea (HU) treatment. (A) Shows cell synchronization at G_1/S phase at the end of HU exposure in the absence of further treatment (CONTROL 0 h) characterized by accumulation of cells exhibiting DNA content of 2n. Four hours after washout of HU a significant portion of cells passed through S phase to G2 exhibiting 4n DNA content (B, CONTROL 4 h). (C) Depicts inhibition of S phase progression by 250 μM GSNO (GSNO 4 h). The reducing agents NAC and GSH (10 mM) prevent inhibition of S phase progression by GSNO (D and E), whilst NAS has no effect (F). Data are representative of four different experiments.

3.2±0.2% of control for 62.5–1000 μM GSNO, respectively, Figure 2A). Higher concentrations (1 mM) of S-nitroso-penicillamine, (SNAP) were required to produce a significant inhibition of DNA synthesis (40.8±14.4% of control at 4–5 h). Flow cytometry also revealed that the nitrosothiols prevented S phase progression (Figure 1C, GSNO). Similarly to S-nitrosothiols, increasing concentrations of a slow releasing NO donor, DETA-NONOate and serial dilutions of saturated solutions of NO gas also inhibited DNA synthesis in a concentration dependent manner (Figure 2B,C). Importantly, the effects of NO donors were prevented by 10 μM haemoglobin or 100 μM cPTIO, a cell permeable NO

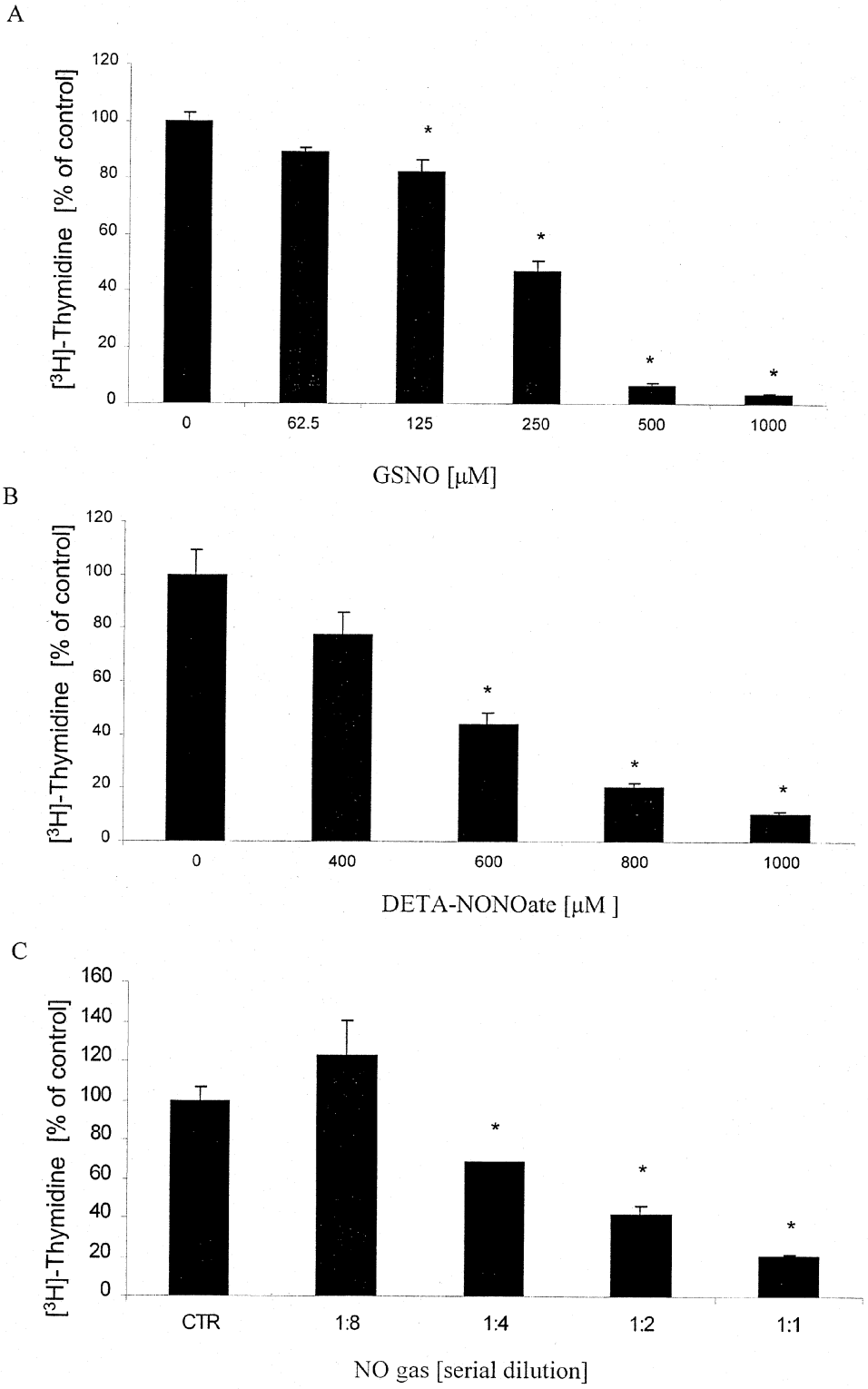

Figure 2 Effects of S-nitrosoglutathione (GSNO), DETA-NONOate and NO saturated medium on DNA synthesis. DNA synthetic rate was monitored by [^{3}H]-thymidine incorporation into acid insoluble macromolecules in cells synchronized at G1/S boundary by 12 h hydroxyurea (HU) treatment. (A) Shows that GSNO inhibits DNA synthetic rate 4 h after washout of HU, in a concentration dependent manner. Higher concentrations of DETA-NONOate were necessary for inhibition of DNA synthesis (B). (C) Shows that serial dilutions of NO saturated medium inhibits DNA synthesis after washout of HU in a concentration dependent manner. Data are mean±s.e.mean from a representative experiment of three; using eight replicates, *=$P<0.05$ from the appropriate control cells in the absence of NO donors.

scavenger, suggesting that they were mediated by NO release (data not shown).

To assess the influence of redox agents on NO-induced inhibition of S phase progression, cells were treated with increasing concentrations of N-acetyl-L-cysteine (NAC) and N-acetyl-L-serine (NAS, a similar compound but lacking the reduced thiol moiety) before addition of the S-nitrosothiols. The inhibition of thymidine incorporation by GSNO (250 μM) was reversed by NAC in a concentration dependent manner (77.6 ± 4.1 and 83.3 ± 1.8% of control in the presence of 5 and 10 mM NAC vs 48.3 ± 6.1% of control in the absence of NAC, Figure 3A). GSH had a similar effect, however NAS (10 mM) was ineffective (42.9 ± 1.6% of control, Figure 3B). These results were substantiated by flow cytometry, which showed that NO-induced inhibition of S phase progression was reversed by 10 mM NAC and GSH (Figure 1D,E), but not by NAS (Figure 1F). [^{3}H]-Thymidine uptake showed that the inhibition of DNA synthesis by SNAP, DETA-NONOate and NO gas was also prevented in a concentration dependent manner (data not shown). To investigate whether the effects of reducing agents were limited to reduced thiols, cells were treated with another reducing agent and antioxidant, L-ascorbic acid. Like the reduced thiols, L-ascorbic acid also prevented the action of NO on DNA synthesis as assessed by [^{3} H]-thymidine uptake (67.4 ± 5.0% of control in the presence of 10 mM L-ascorbic acid vs 19.0 ± 1.3% of control in the absence of L-ascorbic acid), although L-ascorbic acid alone inhibited thymidine uptake (50.1 ± 7.5% of control). Flow cytometry also showed that NO inhibition of S phase progression was prevented by L-ascorbic acid (data not shown).

To explore further the underlying mechanism whereby redox agents modulate the effects of NO on DNA synthesis, we performed a series of experiments to study potential extracellular and intracellular interactions between NO and redox agents including (1) release of NO from NO donors, (2) intracellular bioactivity of NO and (3) reversal of the activity of DNA synthesis by redox agents after established inhibition by NO.

To explore whether the effect of reducing agents on GSNO-induced inhibition of DNA synthesis could be explained by reduced liberation of NO from the nitrosothiols, we monitored

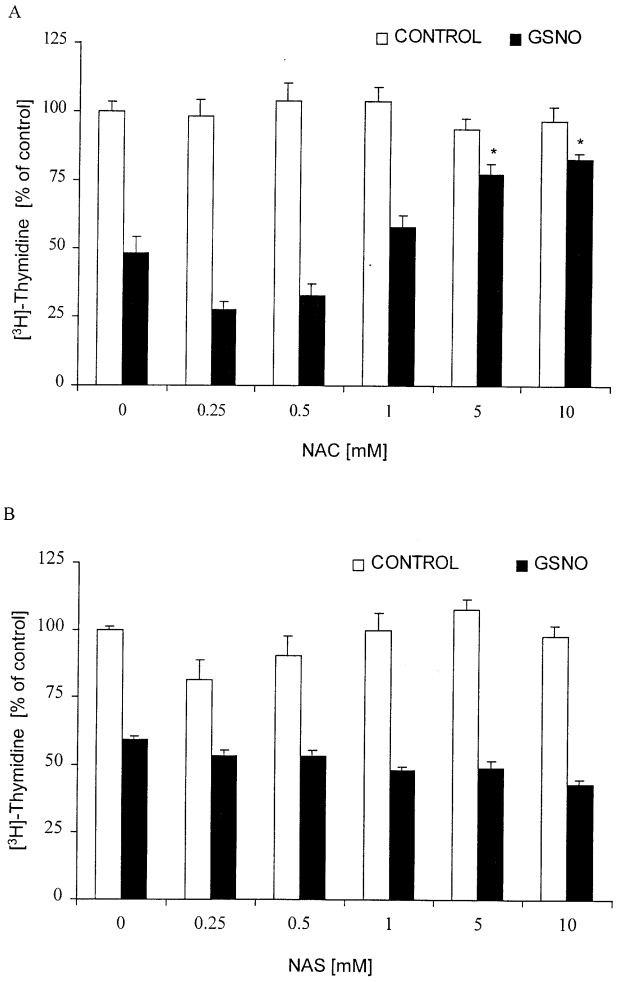

Figure 3 Effect of reducing agent N-acetyl-L-cysteine (NAC) and N-acetyl-L-serine (NAS) on DNA synthesis inhibition by GSNO. DNA synthetic rate was measured by [^{3}H]-thymidine incorporation into acid insoluble macromolecules in human aortic smooth muscle cells that were synchronised at the G1/S boundary by hydroxyurea and then treated with 250 μM GSNO in the presence or absence of increasing concentrations of reducing agents for 4 h. (A) Shows increase in DNA synthetic rate in control cells 4 h after washout of HU, inhibition of DNA synthesis by GSNO (250 μM) and prevention of this inhibition by increasing concentrations of NAC. NAS had no effect in preventing GSNO-induced inhibition of DNA synthesis (B). Data are mean $\pm$ s.e.mean of four separate experiments, using eight replicates each. * = $P < 0.05$ from control cells in the absence of reducing agents.

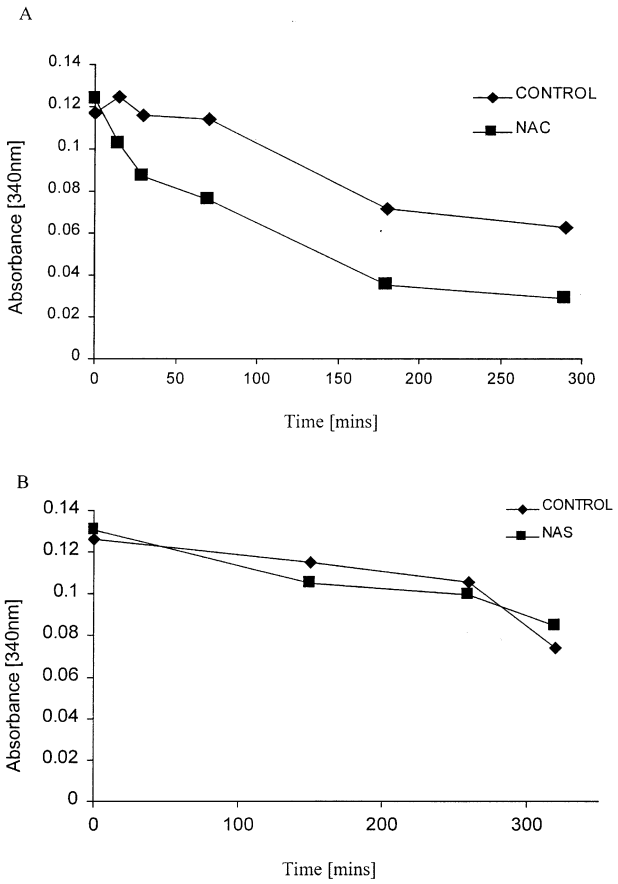

Figure 4 Influence of N-acetyl-L-cysteine (NAC) and N-acetyl-L-serine (NAS) on S-nitrosothiol stability. The specific absorbance of S-nitrosothiols in culture medium was monitored by spectrophotometry at 340 nm. (A) Shows an increased decomposition of GSNO (250 μM) over time in the presence or absence of NAC. (B) Shows that NAS had no influence on the kinetics of GSNO decomposition under similar conditions. Data are a representative experiment of three.

both the stability of the nitrosothiols over time and NO release from the nitrosothiols. The specific absorbance of GSNO at 340 nm decreased in control cells over 4 h (optical densities of 0.063 ± 0.006 vs 0.117 ± 0.006 at 4 h vs 0 h, respectively, Figure 4A). In the presence of NAC this time dependent decrease in absorbance was more pronounced (0.03 ± 0.006 at 4 h, Figure 4A), suggesting accelerated decomposition of GSNO. This was also evident with GSH and L-ascorbic acid, but not with NAS (Figure 4B).

Chemiluminescence analysis showed an immediate increase in NO signal upon addition of 250 μM GSNO, reaching a steady state, signifying a constant rate of release of NO gas from the nitrosothiol (Figure 5A). Addition of NAC caused a further dose-dependent increase in the NO signal. In contrast, NAC did not produce a signal in the absence of GSNO (Figure 5A) and 10 mM NAS did not potentiate NO release from GSNO (Figure 5B). Moreover, 10 mM GSH increased the NO signal, which was further accentuated by addition of 10 mM L-ascorbic acid (Figure 5B).

To evaluate the biological consequences of increased decomposition of nitrosothiols and augmented release of NO by redox agents and to test the specificity of redox agents on NO-induced DNA synthesis, we investigated another intracellular effect of NO by assessing activation of soluble guanylate cyclase and accumulation of cyclic GMP. Treatment of cultured human aortic SMC with increasing concentrations of GSNO for 15 min resulted in no change in cyclic GMP content (22.2 ± 3.9; 25.6 ± 4.4; 28.25 ± 4.5 fmol well^{-1} for control, 250 and 500 μM GSNO, respectively). Since freshly obtained human aorta pieces produced high cyclic GMP levels in response to similar concentrations of NO donors (100 fold increases), it is likely that soluble guanylate cyclase is downregulated in the cultured human cells. To overcome this difficulty, these series of experiments were repeated using rat aortic SMC, a cell line which responds to NO donors by cyclic GMP accumulation. Similarly to human aortic SMC, NO donors inhibited [^{3}H]-thymidine uptake in these cells, and the reducing agents NAC, GSH and L-ascorbic acid prevented this effect. These cells also accumulated cyclic GMP in response to a 15 min exposure to GSNO (112.5 ± 5.6; 156.3 ± 4.8; 218 ± 15.5% of control for 10, 50 and 100 μM GSNO, respectively). GSNO-induced accumulation of cyclic GMP in these cells was not altered by inclusion of 10 mM NAC (194.1 ± 12.1 vs 218.0 ± 15.5% of control for 100 μM GSNO. In addition, GSNO-induced intracellular cyclic GMP accumulation was potentiated in the presence of L-ascorbic acid as the redox active agent (337.1 ± 5.0 vs 116.1 ± 5.9; 362.3 ± 23.5 vs 160.7 ± 11.7; 327.3 ± 29.3 vs 224.6 ± 11.9% of control for 10, 50 and 100 μM GSNO in the presence and absence of L-ascorbic acid, respectively.

To obtain information regarding NO bioactivity over a longer period in a redox active environment, cyclic GMP accumulation was analysed after a 4 h incubation period either in the absence or presence of redox agents. GSNO-induced increases in cyclic GMP levels over 4 h were comparable in the absence or presence of redox agents (Figure 6).

To explore the possibility that redox agents might restore DNA synthesis activity after established inhibition by NO, the kinetics of DNA synthesis reversal was investigated after removal of NO donors followed by incubation either in the presence or absence of redox agents. As before, incubation with GSNO (250 μM for 4 h) resulted in inhibition in DNA synthesis. In these series of experiments, washout of GSNO and incubation in NO donor-free medium resulted in a partial restoration of DNA synthesis over the subsequent 60 min (Figure 7A). The kinetics of DNA synthesis reversal was similar both in the absence or presence of post-treatment with

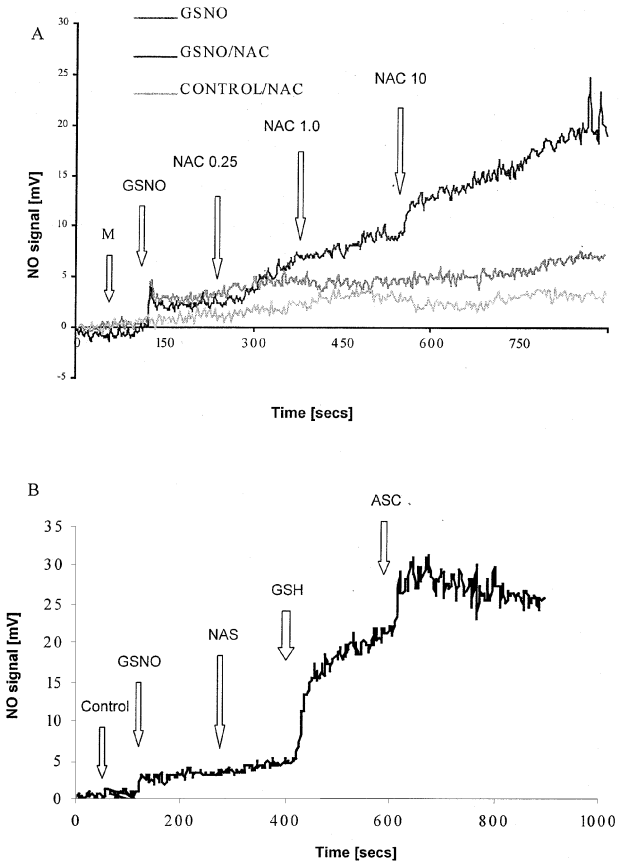

Figure 5 Effect of redox agents N-acetyl-L-cysteine (NAC) (A), glutathione (GSH) and ascorbic acid (ASC) (B) on release of NO from GSNO measured by chemiluminescence. Serum-free culture medium was injected into the chamber of the chemiluminescence analyser and nitrogen gas bubbled through for 30 min to equilibrate. Injection of GSNO (250 μM) produced a peak NO signal that was further increased on injection of NAC (0.25–10 mM) in a concentration dependent manner (A). Injection of NAC (10 mM) in the absence of GSNO gave no NO signal. (B) Shows the NO signal produced by GSNO was also increased on injection of GSH (10 mM) and this was further increased by ASC (10 mM). NAS (10 μM) had no effect. Data are representative of three separate experiments.

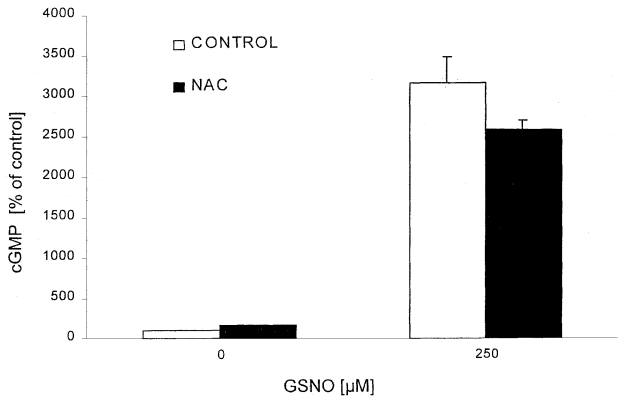

Figure 6 Effect of N-acetyl-L-cysteine (NAC) on GSNO-induced intracellular accumulation of cyclic GMP in rat aortic smooth muscle cells (SMC). Cells were treated with GSNO (250 μM) in the presence and absence of NAC (10 mM) for 4 h. The figure shows that NO-induced cyclic GMP accumulation remains comparable in the absence or presence of NAC over a 4 h incubation. Data are mean $\pm$ s.e.mean of a representative experiment of three; using four replicates.

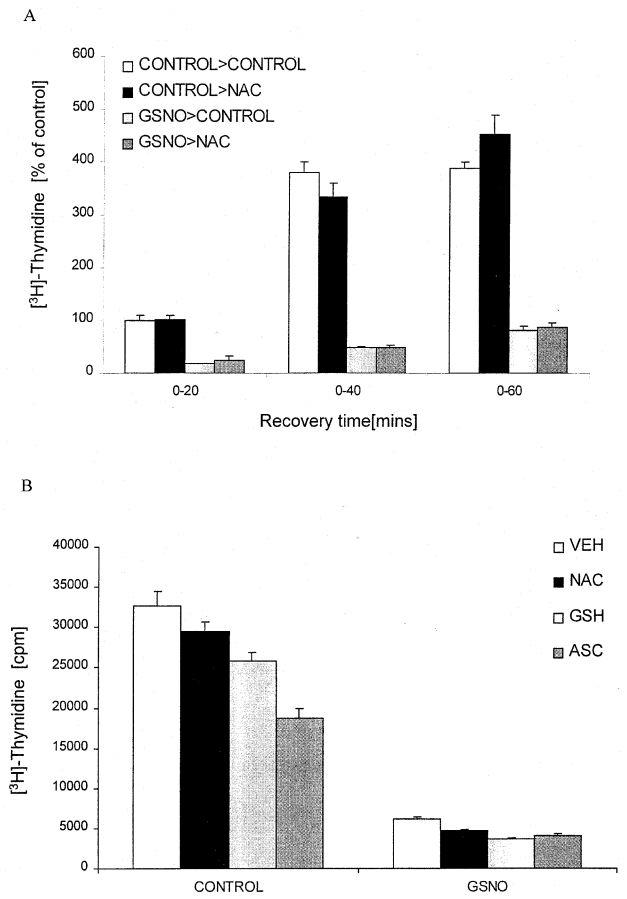

Figure 7 Effect of N-acetyl-L-cysteine (NAC) on reversal of GSNO inhibition of DNA synthesis. Cells were synchronized at the G1/S boundary by 12 h hydroxyurea (HU) treatment and then incubated for 4 h in the absence as control and presence of 250 μM GSNO. The recovery from inhibition of DNA synthesis by GSNO was then analysed by [^{3}H]-thymidine incorporation over the subsequent 60 min in the presence or absence of NAC (10 mM). (A) Shows reduced DNA synthetic rate in GSNO treated cells after washout of GSNO and a similar degree of only partial recovery over 60 min in the absence and presence of NAC. Extending the recovery time to 2 h and using distinct redox agents such as glutathione (GSH) and L-ascorbic acid (ASC, 10 mM) or vehicle (VEH) also demonstrates a lack of effect of these redox agents on the recovery of DNA synthesis after treatment with GSNO (B). Data are mean$\pm$s.e.mean of a representative experiment of three; using six replicates.

10 mM NAC. Extending this time course to 2 h after washout of GSNO and to the other redox agents produced similar results and demonstrates that there remained a significant inhibition in NO-treated cells, which was not influenced by the presence of NAC, GSH or L-ascorbic acid (Figure 7B).

Discussion

These data confirm previous observations regarding NO-induced inhibition of ongoing DNA synthesis and extends these observations to human vascular tissue. The novel finding of the current study is the modulation of NO-induced DNA synthesis inhibition during S phase progression by structurally unrelated redox-active agents in human aortic SMC. We have considered several mechanisms underlying the effects of redox agents and have concluded that redox agents are likely to interfere with specific intracellular mechanisms mediating NO-induced attenuation of ongoing DNA synthesis without inhibiting other intracellular effects of NO such as activation of soluble guanylate cyclase-cyclic GMP pathway.

An alternative explanation for the influence of redox agents would be interference with NO release from NO donors or conversion to inactive metabolites after its release of NO. This possibility was studied by several means. We used chemically different NO donors exhibiting clearly distinct kinetics and mechanisms of NO release and NO gas itself and distinct redox agents to minimize potential chemical interference specific to a particular class of NO donors. With regard to S-nitrosothiols as one of the most frequently used NO donors, we assessed NO release by monitoring both S-nitrosothiol stability and release of NO itself by chemiluminescence. Finally we assessed the biological consequence of the interaction between redox agents and NO donors by determination of intracellular NO bioactivity.

Due to the recognition of the variety of novel biological functions of S-nitrosothiols, there has been increasing activity to delineate the chemical reactivity of these compounds in redox active environment. Transnitrosation appears to be the simplest possible reaction between S-nitrosothiols and thiols where the NO moiety is directly transferred from one thiol to another (Meyer *et al*., 1994; Scharfstein *et al*., 1994). This reaction does not alter the concentrations of the parent compounds and could be involved in NO trafficking. However, recent studies highlight a more complex chemistry between S-nitrosothiols and thiols and suggest multiple series of sequential reactions either increasing or decreasing NO bioactivity with the ultimate formation of a variety of species including nitroxyl, ammonia, nitrous oxide and nitrite (Singh *et al*., 1996b; Stamler, 1995; Wong *et al*., 1998).

Monitoring interactions between S-nitrosothiols and thiols in the cell culture medium in the presence or absence of cells revealed increased decomposition of S-nitrosothiols. This finding confirms previous observations (Singh *et al*., 1996a) and argues against the role of the single transnitrosation reactions since these would lead to regeneration of the S-nitrosothiol. During decomposition of the S-nitrosothiols, NO can either be liberated or consumed by a multitude of secondary reactions. Although we cannot rule out that in the aerobic environment of our cell culture experiments there may be the formation of biologically inactive nitrogen compounds that may also contribute to the prevention of NO effects on DNA synthesis by redox agents, we directly demonstrate increased release of NO from S-nitrosothiols by redox agents. Taken together redox agents increased S-nitrosothiol decomposition and this event was associated with augmented liberation of NO.

To assess the intracellular biochemical consequences of redox agent-NO donor interactions and to evaluate whether prevention of NO action on DNA synthesis was part of an overall inhibition of NO signalling we monitored soluble guanylate cyclase activation by measuring cyclic GMP accumulation. Our data show that as opposed to their effect on NO-induced DNA synthesis, cyclic GMP accumulation remained unaltered or was potentiated by the redox agents. This was not only true for short incubations, but cyclic GMP levels after 4 h incubations were also comparable in the absence or presence of redox agents. These data demonstrate that redox agents liberated biologically active NO from NO donors and support the postulation that the effect of NO on the sGC-cyclic GMP pathway and DNA synthesis are regulated by distinct redox mechanisms (Stamler *et al*., 1989).

Similar to our findings, increased release of NO and cellular levels of cyclic GMP have been reported to potentiate physiological responses to NO and to NO donors such as vasodilatation and platelet aggregation (Stamler *et al*., 1989).

The beneficial effects of ascorbic acid on NO-mediated vasodilatation has recently received considerable interest. It has been found to improve vasodilatation in diabetes, coronary artery disease and in congestive heart failure (Hornig *et al*., 1998; Levine *et al*., 1996; Ting *et al*., 1996). This effect is generally attributed to interference with oxidant stress and increased half-life of NO by ascorbic acid. Our data suggest an additional mechanism to explain the beneficial effects of ascorbic acid in promoting NO release from endogenous nitrosothiols.

In light of increased liberation of NO from NO donors and unaltered cyclic GMP accumulation, the inhibitory effects of redox agents on NO-induced DNA synthesis appears to be a specific intracellular effect. The exact mechanisms of this redox regulation remains to be elucidated. One of the principal targets of NO in inhibiting DNA synthesis in tumour cells appears to be ribonucleotide reductase (Kwon *et al*., 1991; Lepoivre *et al*., 1991). Recently we provided evidence for a similar role of this enzyme in mediating NO-induced inhibition of DNA synthesis in human vascular cells (Bundy *et al*., 1999). Ribonucleotide reductase has been reported to possess a catalytically competent tyrosyl free radical generated by redox sensitive regulatory components including a diiron centre on the R2 subunit and critical thiols on the R1 subunit (Bollinger *et al*., 1991). We hypothesize that these redox targets could be involved in conferring the interaction between redox agents and NO in vascular smooth muscle cells used in our study. Redox agents might prevent NO-induced modulation of these centres or might restore the optimal redox state of these centres after NO attack. This latter hypothesis could be indirectly addressed in the present study. However, we did not find any evidence for increased recovery of DNA synthesis after NO inhibition by post-treatment with redox agents.

Our observations could have important physiological and clinical implications regarding the control of vascular smooth muscle cell proliferation by endogenous NO and S-nitrosothiols. There is compelling evidence that NO produced constitutively or *via* induced NO synthase during inflammatory conditions plays a significant role in preventing intimal hyperplasia (McNamara *et al*., 1993). Our data suggest that this effect might be substantially modulated by the redox environment. Since reduced thiols are present in significantly high millimolar concentrations in mammalian cells, these interactions could represent important physiological regulatory mechanisms in growth control by NO.

The results of this study could also have some therapeutic potential. High concentrations of NO have been reported to cause DNA damage and mutagenesis (Nguyen *et al*., 1992). An effect on DNA synthesis and cellular proliferation might also complicate clinical therapy *via* NO inhalation or by NO donors. Our findings suggest that drugs currently used in clinical practice, such as NAC or ascorbic acid could prevent these DNA synthesis related side effects and apparently potentiate the cyclic GMP-mediated vasodilatation.

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