S-nitrosothiols inhibit uterine smooth muscle cell proliferation independent of metabolism to NO and cGMP formation

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S-nitrosothiols (RSNOs) have received much attention as possible mediators of NO function in vivo (11, 19, 47). Specific metabolic pathways in cells that reduce RSNOs, forming either NO (1-electron reduction) or nitroxyl anion (HNO; 2-electron reduction), can then activate sGC-inducing responses such as vasodilation, have been proposed (2, 16, 19–21, 47). However, it is becoming more evident that other reaction mechanisms, independent of RSNO reduction, involving S-nitrosation or S-thiolation of specific protein thiols mediate the biological actions of RSNOs (19, 48, 55).

Interestingly, NO-dependent signaling pathways that regulate similar cellular functions have been identified that are both sGC dependent and independent (7, 13, 42, 43). It is not clear, however, whether RSNOs are important mediators of sGC-independent pathways. One such process is the antiproliferative functions of NO toward a number of cell types including vascular and mesangial smooth muscle cells (8, 42). In the vasculature this is thought to be an important mechanism by which NO prevents the neointimal hyperplasia observed after damage to the vessel wall that occurs during balloon angioplasty (23, 43). A relatively unexplored area of this aspect of NO biology, however, is the effect on uterine smooth muscle cell function. In the uterine and feto-placental environments, there is potential for exposure to high concentrations of NO through maternal serum, amniotic fluid, and contact with immune cells and decidua (1, 9, 17, 24, 36, 51, 53). In the context of vasodilatation, it is interesting to note that the NO-dependent signaling mechanisms that are present in the vasculature do not mediate uterine smooth muscle relaxation (51).

Differenciated adult smooth muscle cells normally have a low rate of proliferation. However, uterine smooth muscle cell growth occurs during pregnancy.
Abnormal proliferation of uterine smooth muscle cells may contribute to formation and progression of benign myometrial tumors known as leiomyomata (3, 39). In addition, hyperplasia of junctional zone (subendometrial) myometrial smooth muscle cells is a common finding in women with menstrual dysfunction (10). Although both physiological and pathophysiological consequences of smooth muscle cell growth have been established in the uterus, very little is known about the mechanisms controlling cell proliferation and whether or not NO and cGMP are involved. Several studies have demonstrated that in the context of relaxation, the NO-cGMP pathway is downregulated during pregnancy (10, 36, 49, 51). These observations illustrate the dynamic regulation of NO-dependent pathways in the uterus. Consistent with these concepts, the effect of NO is modulated by the hormones estrogen and progesterone by increasing or decreasing PKG, respectively (9).

A factor not considered previously, however, is the potential role of NO through RSNOs in mediating uterine smooth muscle cell function under conditions in which the cGMP pathway is downregulated. The current study was undertaken in this context to examine the antimitogenic role of NO and RSNOs in uterine smooth muscle cells. We tested the hypothesis that RSNOs inhibit proliferation of uterine smooth muscle cells and that this effect is not mediated by the classic NO-cGMP pathway. In the rat uterine smooth muscle cell line ELT-3, our data indicate that RSNOs are metabolized to NO by ELT-3 cells and inhibit uterine smooth muscle cell proliferation, but this effect is not mediated by the NO-cGMP pathway.

EXPERIMENTAL PROCEDURES

Preparation and culture of uterine smooth muscle cells. Preparation of the continuous rat uterine cell line ELT-3, isolated from an Eker rat leiomyoma, was described previously (12). ELT-3 cells retain a smooth muscle phenotype based on histological appearance and immunological criteria including positive staining for α-smooth muscle actin and desmin. Cells were grown on plastic culture dishes or flasks in Ham’s F-12-Dulbecco’s modified Eagle’s medium (DMEM) (50:50) containing 1.6 × 10⁻⁶ M ferrous sulfate, 1.2 × 10⁻⁵ U/ml vasopressin, 1.0 × 10⁻⁹ M triiodothyronine (T₃), 2.0 × 10⁻⁷ M hydrocortisone, and 10 µg/ml transferrin (DF-8) supplemented with 10% fetal bovine serum (FBS). For ³H-labeled thymidine incorporation experiments, cells were plated at 2.0 × 10⁵ cells/cm² in 24-well plates and serum deprived for 48 h when just confluent in DF-8 medium lacking vasopressin and containing 0.5% FBS. For radioimmunoassays, cells were grown to confluence and medium was changed to a basic salt solution. Cells were incubated for 5 min to 3 h in the presence or absence of 0.5 mM IBMX at 37°C in an air environment. Experiments were undertaken when cells were confluent, usually within 1 wk of plating in a humidified 5% CO₂ atmosphere at 37°C.

Treatment of cells with NO gas. A saturated solution of NO was made by bubbling deionized water with argon for 15 min in a closed system with greased vacuum joints, followed by bubbling with NO gas for 15 min. All gases flowed through a trap containing NaOH pellets, followed by a trap containing 10 M NaOH. The concentration of NO was verified with an NO electrode (WPI) and comparison to standards as supplied by the manufacturer.

³H/thymidine incorporation. Cultures were serum deprived for 48 h before treatment with 5% FBS or 10–20 ng/ml PDGF-BB in the presence or absence of NO donors or cGMP analogs. Twenty hours after stimulation, ³H-thymidine was added (10 µCi/ml). Four hours later, the medium was aspirated and cells were fixed and permeabilized by addition of 0.2 ml of methanol-acetic acid (3:1) for 1 h. Wells of ELT-3 cells were then washed twice with 80% methanol. The remaining cell material was solubilized with 0.25 ml of 0.1 N NaOH and transferred to scintillation vials. The wells were washed with an equal volume of 0.1 N HCl, and this was added to the vials. Incorporated ³H-thymidine was measured by scintillation spectroscopy. Data are reported as mean ± SE counts per minute of ³H-thymidine incorporated per well or as a percentage of stimulation by growth factor.

cGMP radioimmunoassay. cGMP was determined by radioimmunoassay with the procedure of Brooker et al. (4). Cells were plated in 35-mm dishes and treated with NO donors. After treatment, the medium was aspirated and cells were placed on ice. The cells were washed once quickly with 1.25 ml of ice-cold phosphate-buffered saline (PBS) and then extracted with 0.75 ml of 50% methanol-0.1 N HCl. The acidified methanol extracts were lyophilized and processed for cGMP radioimmunoassay.

S-nitrosogluthathione metabolism and NO formation. Cells were grown to confluence in 6- or 12-well plates in phenol red-free DF-8 medium. At the time of the experiment medium was aspirated and phenol red-free DF-8 with S-nitroso-gluthathione (GSNO) was added to the cells and cell-free wells. At various time points 1 ml of medium was removed from each well and the ultraviolet light (UV) absorbance at 337 nm (ε₃₃₇nm) was recorded to determine the extent of GSNO consumption. GSNO concentrations were calculated with ε₃₃₇nm = 900 M⁻¹ cm⁻¹. After the last time point oxymyoglobin (20 µM heme) was added to each well to determine the rate of NO production. Oxymyoglobin reacts with NO with a stoichiometry of 1:1, forming metmyoglobin. This change is accompanied by a change in the visible spectrum of myoglobin, allowing its quantitation. It should be noted that with this method and under aerobic conditions, reactions between oxymyoglobin and the nitrosyl anion (HNO) will also lead to the formation of metmyoglobin. Because RSNOs can be reduced by 1 or 2 electrons, forming NO and HNO, respectively, this protocol was used to determine the role of RSNO reduction on the antiproliferative effects of GSNO.

After 15–30 min of incubation with oxymyoglobin, 1 ml was removed from each well and the absorbance at 582 and 524 nm was recorded and loss of oxymyoglobin (and hence NO) was measured with this method and under aerobic conditions, reactions between oxymyoglobin and the nitrosyl anion (HNO) will also lead to the formation of metmyoglobin. Because RSNOs can be reduced by 1 or 2 electrons, forming NO and HNO, respectively, this protocol was used to determine the role of RSNO reduction on the antiproliferative effects of GSNO. After 15–30 min of incubation with oxymyoglobin, 1 ml was removed from each well and the absorbance at 582 and 524 nm was recorded and loss of oxymyoglobin (and hence NO production) was determined by using ε₅₈₂ₛ₅₄nm = 14.4 M⁻¹ cm⁻¹. Absorbance measurements at 582 nm were normalized to the absorbance at 524 nm, an isosbestic point for the oxymyoglobin-metmyoglobin transition. Also, oxymyoglobin was added to cell-free wells and absorbance changes due to background autooxidation (which also generates metmyoglobin) were subtracted from changes observed in the presence of cells.

Data for experiments involving GSNO metabolism and RIA for cGMP were normalized to cell protein. Protein was determined with the Bradford assay and bovine serum albumin as standard.

Materials. FBS, DMEM, antibiotics, and other culture reagents were from GIBCO (Grand Island, NY). (N,N-diethylamino)-diazenolate-2-oxidiediethylammonium (DEA-NONOate) and (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate (DETA-NONOate) were obtained from Alexis.
Biochemicals; Angelis salt was purchased from Cayman Chemicals. S-nitroso-N-acetyl-penicillamine (SNAP) was purchased from Research Biochemicals International (Natick, MA). 125I-labeled cGMP was from New England Nuclear Dupont (Boston, MA). Horse heart myoglobin and all other medium supplements and reagents were from Sigma (St. Louis, MO) or Fisher Biochemicals (Pittsburgh, PA).

RESULTS

Inhibition of [3H]thymidine incorporation by S-nitrosothiols. Previous studies showed that NO donors including RSNOs inhibit vascular smooth muscle cell proliferation. We therefore tested the effects of the low-molecular-weight RSNOs GSNO and SNAP on [3H]thymidine incorporation in a uterine smooth muscle cell line (ELT-3). ELT-3 cells were stimulated with growth factors [PDGF or bFGF (basic FGF)] as described in EXPERIMENTAL PROCEDURES, in the presence of varying concentrations of SNAP or GSNO (Fig. 1). Both GSNO and SNAP inhibited DNA synthesis in a concentration-dependent manner as shown by IC50 values of ~40 and 100 µM, respectively. Similar effects of RSNOs on cell proliferation were obtained when cells were counted directly by light microscopy (not shown). These values are similar to those reported for inhibition of [3H]thymidine incorporation in vascular cells, although two to three orders of magnitude higher than the concentrations required to achieve maximal relaxation of vascular smooth muscle. Identical results were found in human uterine smooth muscle cells in primary culture through passage 3 treated with SNAP (data not shown). Because GSNO was more efficient at inhibiting ELT-3 proliferation and may be formed in vivo, subsequent studies were conducted with this RSNO.

Effects of GSNO, oxidized glutathione, reduced glutathione, and nitrite on [3H]thymidine incorporation in uterine smooth muscle cells. To ensure that the effect of GSNO on ELT-3 proliferation was due to the nitroso (SNO) moiety, the effects of nitrite (NO2) and reduced (GSH) or oxidized (GSSG) glutathione at concentrations that could maximally be produced (as reaction products) from GSNO were determined. Table 1 shows that NO2 does not inhibit, but slightly increases, [3H]thymidine incorporation. Figure 2 shows the effects of GSNO, GSSG, and GSH on [3H]thymidine incorporation in ELT-3 cells. bFGF increased [3H]thymidine incorporation by four- to fivefold, which was inhibited by ~70% by GSNO. GSSG (250 µM) and GSH (500 µM) had no effect, suggesting that NO was critical for the inhibition of cell proliferation. GSNO, GSH, and GSSG had no effect on cell proliferation in the absence of bFGF.

Rate of GSNO metabolism by ELT-3 cells. Previous studies showed that different cell types have specific, transition metal ion-dependent or -independent, reductive pathways that reduce RSNOs (16, 55). This may occur by one or two electrons, forming NO and HNO, respectively. To test the hypothesis that reduction of RSNOs is critical in the observed inhibition of ELT-3 proliferation, we first examined the metabolism of

![Fig. 1. Effect of S-nitrosogluthathione (GSNO) and S-nitroso-N-acetyl-penicillamine (SNAP) on ELT-3 cell proliferation. ELT-3 cells were serum starved and incubated with PDGF-BB (20 ng/ml) with increasing concentrations of GSNO (●) and SNAP (○) for 20 h. [3H]-labeled thymidine (10 µCi/ml) was then added, and incorporation was measured after 4 h. Data represent means ± SE (n = 3–6).](Image)

![Fig. 2. Effects of GSNO, oxidized glutathione (GSSG), and reduced glutathione (GSH) on ELT-3 cell proliferation. ELT-3 cells were incubated with FGF (10 ng/ml) for 20 h in the presence or absence of GSNO (500 µM), GSH (500 µM), or GSSG (250 µM). [3H]thymidine (10 µCi/ml) was then added, and incorporation was measured after 4 h. Data represent means ± SE (n = 3–6). cpm, Counts per minute.](Image)
GSNO by these cells. Metabolism of GSNO was measured by determining the amount of RSNO remaining after different times of incubation with ELT-3 cells and measuring formation of NO/HNO with oxymyoglobin as a trap. Figure 3 shows that the rate of GSNO metabolism in the presence of cells was higher compared with basal degradation in the medium alone. Interestingly, the addition of the metal chelator diethylenetriamine pentaacetic acid (DTPA) inhibited only basal degradation of GSNO (i.e., decomposition in medium alone). No effect of DTPA on cell-mediated decomposition (calculated by subtracting GSNO decomposition rates in the medium alone from cell-dependent rates) was observed. Figure 4 shows the maximal rate of RSNO decomposition by ELT-3 cells as a function of GSNO concentration. Over the concentration range studied, the rate of RSNO decomposition by ELT-3 cells is higher compared with basal degradation in the medium alone.

Concomitant with measurements of GSNO decay, oxymyoglobin was used to probe the role of reductive reactions in this process. Under aerobic conditions, this heme protein reacts rapidly with NO and HNO, forming metmyoglobin, with no reaction between the heme and GSNO occurring unless the protein is deoxygenated (46). Figure 5A shows that the predominant fate of GSNO added to ELT-3 cells is reduction to either NO or HNO with an approximate yield of 70%. To ensure that the metabolism of GSNO by ELT-3 cells was a physiologically relevant cell-mediated effect and not an artifact of cell transformation or culture, primary cultures of both rat and human uterine smooth muscle cells were analyzed. GSNO metabolism and NO formation were assessed as for ELT-3 cells. Whereas normal rat uterine smooth muscle cells metabolized GSNO in a manner indistinguishable from that of ELT-3 cells (not shown), human uterine smooth muscle cells metabolized GSNO at a rate approximately four times faster (Fig. 5B). Furthermore, treatment of primary cultures of human uterine smooth muscle cells with 20 ng/ml 17β-estradiol for 4 days (hormone added fresh every 24 h) had no effect on the metabolism of GSNO. Estrogen responsiveness of human uterine smooth muscle cells was confirmed by detection of increased levels of progesterone receptor by Western blot analysis (data not shown).

Effects of Angelis salt on [3H]thymidine incorporation in uterine smooth muscle cells. To test the possible involvement of HNO, we investigated the effects of the HNO donor Angelis salt on serum-dependent [3H]thymidine incorporation. Table 1 shows the percentage increase in [3H]thymidine incorporation relative to serum-starved cells. In contrast to GSNO, Angelis salt at concentrations up to 500 μM modestly increased proliferation. Over the concentration range tested, no effects of cell viability were observed (not shown). Increasing the concentration of Angelis salt up to 1 mM, however, did result in cytotoxicity precluding measurement of proliferative responses. These data suggest that HNO is not a primary intermediate in the mechanisms by which GSNO inhibits ELT-3 cell proliferation.

Effect of NO on [3H]thymidine incorporation in ELT-3 cells. To test directly the hypothesis that NO is mediating the observed effects of GSNO, NO gas and NO donors were added to ELT-3 cells and effects on [3H]thymidine incorporation were determined. Two NO donors, DEA-NONOate and DETA-NONOate, were selected because of their spontaneous yet different rates of NO release in solution at pH 7.4. DEA-
NONOate rapidly releases NO with a reported half-life on the order of minutes, whereas DETA-NONOate decomposes with a half-life of \(10^{-2} \text{ h}\). These NO donors are therefore useful tools to simulate conditions of NO exposure that cells are likely to experience under basal (i.e., relatively low rates of NO formation) and inflammatory (high rates of NO formation) states in vivo. Interestingly, Fig. 6A shows that addition of DETA-NONOate or DEA-NONOate over a wide concentration range (0–100 \(\mu\)M) did not inhibit growth factor-induced ELT-3 cell proliferation. Furthermore, addition of NO gas (0–10 \(\mu\)M) also failed to inhibit FGF-stimulated ELT-3 cell growth (data not shown).

Effect of atrial natriuretic peptide and cGMP on \([\text{^3H}]\)thymidine incorporation in uterine smooth muscle cells. To further study the involvement of the classic NO, cGMP, and PKG signaling pathway in mediating NO-induced inhibition of DNA synthesis, the effects of atrial natriuretic peptide (ANP) II and cGMP analogs were tested. ANP II (0–100 \(\mu\)M), an NO-independent activator of guanylate cyclase, at concentrations that stimulate cGMP formation (see below and Fig. 7) failed to inhibit ELT-3 proliferation. Three cGMP analogs were also tested, 8-bromo-cGMP (8-BrcGMP), 8-(4-chlorophenylthio)cGMP (CPT-cGMP), and 8-bromo-\(\beta\)-phenyl-1,\(\beta\)-etheno-cGMP (8-Br-PET-cGMP). Although 8-BrcGMP may undergo metabolism by phosphodiesterases, in general these analogs are more cell permeant and phosphodiesterase resistant than cGMP itself. The CPT and PET compounds activate PKG I with \(K_a\) values of 25 or 50 nM, respectively, and both activate PKG I with a \(K_a\) of 25 nM. 8-BrcGMP had no effect on DNA synthesis in ELT-3 cells (Fig. 6B) and human uterine smooth muscle cells (data not shown), even at 1 mM. Likewise, CPT-cGMP did not inhibit...
PDGF-stimulated DNA synthesis in ELT-3 cells. 8-Br-PET-cGMP at the highest concentration tested only modestly (~20%) inhibited [3H]thymidine incorporation.

**Levels of cGMP in uterine smooth muscle cells treated with NO donors and S-nitrosothiols.** Finally, to test whether the inhibition of uterine smooth muscle cell DNA synthesis by RSNO donors is mediated through cGMP formation, levels of this cyclic nucleotide were determined. After a 3-h incubation in the presence of IBMX, little or no cGMP was produced by GSNO, SNAP, sodium nitroprusside (SNP), DEA-NONOate, or DEA-NO (Fig. 7). Of all compounds tested, only ANP II was capable of increasing levels of cGMP (Fig. 7). However, even with the >100-fold elevation in cGMP levels, ANP II did not inhibit the incorporation of [3H]thymidine in ELT-3 cells (Fig. 6A).

**DISCUSSION**

Cellular responses to NO are diverse and putatively involve both NO- and RSNO-dependent mechanisms. Specific examples of RSNO-mediated regulation of protein function have been reported and are important in diverse cellular processes including those that mediate cell survival and death (19). An important cytostatic and anti-inflammatory role of NO is inhibition of smooth muscle cell proliferation and hypertrophy. Most studies in this area of NO biology have focused on the effects on vascular smooth muscle, and important insights into the inhibition by NO of neointima formation after endothelial injury have been gained (8, 13, 23, 43).

During pregnancy, uterine cells undergo hypertrophy and hyperplasia. We hypothesized that the NO-cGMP signaling pathway inhibits uterine smooth muscle cell growth, thereby providing a reason for the downregulation of this pathway during pregnancy (49). In addition, RSNOs have been identified in vivo and may serve to prolong the half-life of NO and allow it to work at a site distant from the site of synthesis. The precise mechanisms by which RSNOs are formed in vivo remain unclear, although reaction between NO and O2, particularly in hydrophobic compartments and when NO is synthesized in relatively high concentrations, is one possible mechanism (28, 44). Because NO is generated in relatively high concentrations in certain compartments during pregnancy (53), it is feasible that formation of RSNOs can occur and modulate uterine smooth muscle growth. We tested these hypotheses by investigating the antiproliferative effects of both RSNOs, which can mediate both NO-dependent and -independent effects, the NONOate compounds, which release only NO, and NO gas itself, on growth factor-dependent ELT-3 cell proliferation. S-nitrosoglutathione, a biologically relevant low-molecular-weight RSNO, inhibited ELT-3 proliferation. Surprisingly, neither NO gas nor both slow and fast NO-releasing NONOates inhibited ELT-3 cell proliferation. These data suggest that RSNOs inhibit proliferation of uterine smooth muscle cells by a mechanism that does not involve release of NO. Interestingly, the addition of NO gas and the use of DEA-NO produce conditions that would favor formation of RSNOs. However, unlike the direct addition of GSNO or SNAP, no effects on proliferation were observed with DEA-NO or NO gas. This may represent differences in the site of nitrosative chemistry. For example, because of the high diffusivity and hydrophobicity of NO, it is possible that different cellular thiols are targeted by NO compared with addition of RSNOs, which are likely to react with cell surface thiols. These possibilities remain to be investigated.

Further evidence against the classic NO-cGMP pathway for inhibition of uterine smooth muscle proliferation was provided by measuring cGMP in response to NO and use of cGMP analogs. The NO donors, RSNOs, and cGMP analogs did not increase cellular levels of cGMP. This was the case even in the presence of the nonspecific phosphodiesterase inhibitor IBMX. It is therefore unlikely that inhibition of proliferation is occurring via the cGMP-PKG or cAMP-PKA pathway. It is important to note, however, that the cGMP pathway is functional in these cells, as treatment with ANP II significantly elevated cGMP levels (Fig. 7). Our data also highlight the concept that a lack of an effect of cGMP does not preclude a role for NO in mediating biological functions. Furthermore, similar effects were observed with primary rat and uterine smooth muscle cell cultures, indicating that this observation is not specific to the cell line transformation. Interestingly, the human cells were approximately fourfold more efficient at metabolizing GSNO compared with rat uterine smooth muscle cells, although the basis for this difference remains unknown. The RSNO concentrations required to inhibit proliferation are relatively high (~2–4 orders of magnitude) compared with those detected in the circulation (30). However, the concentrations of RSNOs in the uterus are not known and the recently proposed hypothesis that RSNOs may be synthesized locally at high concentrations in hydrophobic compartments does not preclude a role for these species in modulating uterine smooth muscle function (28, 37, 44). Furthermore, the data presented in this study also highlight the therapeutic potential of RSNOs over other NO donors in the treatment of pregnancy-related disorders.

The molecular mechanisms that account for the lack of cGMP pathway responses toward proliferation in uterine smooth muscle cells are not yet known. There has been no clear consensus on a single mechanism of growth inhibition by NO, and it is likely that multiple reaction pathways are involved (7, 34, 42, 43). Early studies demonstrated that NO can prevent DNA synthesis by inhibiting the enzyme ribonucleotide reductase (26). Specifically, this process involves reaction between NO and a protein-bound tyrosyl radical that is important in the catalytic activity of the enzyme. Although RSNOs were reduced by uterine smooth muscle cells, the NONOate compounds that release NO did not inhibit proliferation. These data suggest that inhibition
of ribonucleotide reductase cannot account for RSNO-mediated inhibition of proliferation in this study.

In addition, NO is reported to inhibit other stages of the cell cycle including early and late G1 phase (43). These antiproliferative mechanisms are thought to be mediated by the NO-dependent activation of cGMP; and subsequent production of PKG. This is the same pathway that leads to relaxation in smooth muscle cells. The stage at which RSNOs inhibit ELT-3 proliferation was not examined in this study, but it is clear that the cGMP pathway is not involved. These data demonstrate that different mechanisms for regulating NO bioactivity are present in cell types that are similar but from different tissues, and they underscore the tissue specificity in the responses observed.

Interestingly, the NO and cGMP signaling pathway in uterine smooth muscle has been shown to be regulated by estrogen and progesterone (51). Pregnancy and progesterone administration appear to reduce responsiveness to NO donors and cGMP analogs, whereas tissues from estrogen-dominated animals are more responsive. Similarly, rat uterine smooth muscle responded best to ANP under estrogen domination and poorly during pregnancy and progesterone administration. However, in preliminary studies we did not observe any evidence that estrogen affects NO donor-dependent formation of cGMP (T. L. Cornwell, unpublished observations), and, consistent with the conclusions from this study, estrogen did not affect GSNO metabolism by uterine smooth muscle cells (Fig. 5).

Several studies have demonstrated the ability of specific cell types to reduce RSNOs in a metal-dependent manner (16, 55). Consistent with these reports, the major fate (~60–70%) of RSNOs added to ELT-3 cells was reduction in a process that was insensitive to the metal-chelating agent DTPA. The decay of GSNO was nonsaturable up to millimolar concentrations and is similar to vascular endothelial cell-dependent metabolism of GSNO (55). Interestingly, primary human uterine smooth muscle cells were more effective at reducing GSNO. However, because the observed inhibition of cell growth does not appear to be mediated by either NO or HNO, we did not investigate the nature of these effects further in this study. Possible decomposition products of GSNO metabolism, GSH, GSSG, or NO\textsubscript{2}, had no effect of ELT-3 cell proliferation (Table 1 and Fig. 2), indicating that biological effects required the S-nitroso group. Possible mechanisms for the antiproliferative effects of RSNO include S-nitrosation of a cell target, modulation of a cellular protein via trans-nitrosation, and/or an S-thiolation mechanism (29, 38). These processes do not require production of NO, but they involve direct reaction between the donor RSNO and thiol residues on the target protein. The proposed mechanisms for these effects include RSNO-dependent nitrosation (addition of NO\textsuperscript{+}) or oxidation of a protein thiol to form the corresponding protein-S-nitrosothiol or disulfide. These pathways are gaining importance, and specific examples including protein disulfide isomerase have been identified as potential extracellular targets of RSNO reactivity (38, 54).

An NO-independent effect of RSNOs on uterine smooth muscle function has been inferred in other studies. For example, whereas S-nitrosocysteine inhibited spontaneous monkey uterine smooth muscle cell contractility, cGMP analogs did not (25). However, the mechanisms of these effects were not identified.

It is important to note that many of the studies that have reported the antiproliferative effects of NO toward vascular smooth muscle have used both high-and low-molecular-weight RSNOs as NO donors. However, in these studies evidence has also been presented that the effects observed are mediated by cGMP-dependent mechanisms and/or can be reproduced with the NONOate compounds (7, 8, 13, 23, 34, 42), although differences between poly-S-nitrosated albumin and DETA-NONOate in the ability of either NO donor to inhibit growth of human coronary artery smooth muscle cells were noted by Ewing et al. (13). These data may represent differences in NO release rates from either compound in this cellular system. To our knowledge, the data reported here are the first to demonstrate a RSNO-dependent, but NO- and cGMP-independent, mechanism of inhibiting smooth muscle cell proliferation.

The idea that the NO-cGMP signaling pathway could be regulated in hormonally responsive uterine smooth muscle has been investigated by a number of laboratories. Activators of this pathway are capable of inhibiting contractile force generated by uterine smooth muscle, leading to the hypothesis that pharmacological manipulation of this pathway could be used in the treatment of preterm uterine contractions. However, pharmacological analysis indicates that for the NO-cGMP pathway to relax uterine smooth muscle, high concentrations of NO are required (51). Clinical reports describe the use of nitroglycerin (GTN) or amyl nitrate to relax uterine smooth muscle, and trials using GTN patches to treat preterm labor have been carried out (6, 27). In vitro experiments measuring uterine smooth muscle strip contractility have produced results that vary from lab to lab (35, 51, 53). Because NO donors and cGMP analogs can relax uterine smooth muscle, it has been concluded that the tissue is responsive to this pathway. The data presented herein, however, suggest that the NO-cGMP pathway is not responsive in the context of inhibition of proliferation. Our data, together with the observations that supraphysiological concentrations of NO are required to elicit uterine smooth muscle cell relaxation, indicate that alternative pathways are responsible for the effects of NO in these cells. Elucidation of these pathways will provide important insights into pregnancy and specifically the potential therapeutic use of NO donors.

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