Reduced glutathione prevents nitric oxide-induced apoptosis in vascular smooth muscle cells

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Abstract

The control of medial and neointimal growth, in which vascular smooth muscle (VSM) plays a central role, is most important to the development of hypertension and atherosclerosis, respectively. Growth of vascular smooth muscle cells is regulated by a number of factors, including the vasodilator nitric oxide (NO). In addition, NO modulates intracellular thiol redox states and the thiol redox state of the cell influences NO production. We, therefore, examined the nature of the effect of NO on growth of VSM cells and its modulation by cellular glutathione content. Here, we report that NO, either generated by NO donors or synthesized by iNOS in VSM cells, inhibited DNA synthesis and induced apoptosis in this cell type. NO-induced apoptosis was associated with a significant decrease in the intracellular concentration of reduced glutathione and with an increase in the level of the tumor suppressor gene p53 mRNA. Moreover, addition of glutathione monoethylester to the culture restored the level of reduced glutathione in VSM cells, and prevented the NO-induced increase in p53 expression and programmed cell death. Our findings suggest a role for reduced glutathione in protecting VSM cells exposed to NO from apoptosis. © 1997 Elsevier Science B.V.

Keywords: Glutathione; Nitric oxide; Apoptosis; Vascular smooth muscle

1. Introduction

Growth control of vascular smooth muscle (VSM) within the arterial wall is critical for the development of pathological processes, including hypertension and atherosclerosis [1,2]. Growth of VSM cells is regulated by a number of factors, including transforming growth factor-β (TGF-β), cGMP, heparin and agents that alter the levels of intracellular cGMP [3,4]. Increased levels of cGMP in VSM cells and cell growth arrest can be evoked by nitric oxide (NO) [5]. NO production, in turn, can modulate cell thiol redox state and changes in thiol redox state can affect NO production [6–9]. Growth of cells depends on completion of a full mitotic cell cycle and on rates of cell death. Cell death in aorta in vitro and in vivo has been demonstrated by DNA accumulation rates that were inconsistent with cell mitosis rates [10]. These observations led us to re-examine the nature of the effects of NO on VSM cell proliferation, focusing on programmed cell death (apoptosis) and on its regulation by the cell redox state.

Apoptosis differs from necrosis which occurs when cells are traumatically injured or are subject to inflammatory processes. Apoptosis displays unique morphological changes, including DNA fragmenta-
tion, condensation of cytoplasm, nuclear fragmentation, and budding of cell fragments [11,12]. Apoptosis is induced in different cell lineages by members of the tumor necrosis factor (TNF) family, TGF-β and neurotransmitters, or by growth factor withdrawal. Inhibitors of apoptosis include the CD40 ligand, estrogens, and several viral genes [13]. The mechanism by which these agents affect programmed cell death has been the subject of increasing studies. Certain oncogenes, myc, rel, or E1A, and the tumor suppressor gene p53 have been linked to induction of apoptosis by these agents, depending on the cell type tested [14–16]. The gene Bcl-2 [17,18], however, has been linked to inhibition of apoptosis. Overexpression of Bcl-2 specifically prevents cells from initiating apoptosis in response to a number of stimuli [15,8,19].

NO has been linked to the induction of apoptosis in some cell types [13] and NO production is intimately linked to cellular glutathione content and redox state [7–9]. In the current study we demonstrate that NO induces p53 and activates programmed cell death in VSM cells and that these effects depend on an NO-induced decrease of intracellular reduced glutathione. NO-induced apoptosis may be important for arterial remodeling following vascular injury or in other pathologic states in which vascular smooth muscle cell proliferation is altered.

2. Materials and methods

2.1. Cell culture

Smooth muscle cells were isolated from the thoracic aorta of Sprague–Dawley rats (Charle River Breeding Laboratories). Isolation procedures, using enzymatic disaggregation of tissue, and culturing conditions were described previously [20,21]. Cells were used for experiments between passage numbers 3–6. Cell number and viability were determined with a hemocytometer and staining with trypan blue, respectively.

2.2. Determination of DNA synthesis and cell number

Vascular smooth muscle cells were seeded in 25 cm² flasks at concentrations of 0.5 × 10⁶ cells/5 ml medium. Duplicate flasks were either subjected to cell counting or to pulse labeling for determination of DNA synthesis. In order to determine cell number the cells were scraped off the plate in the presence of phosphate buffered saline (PBS) (10 mM sodium phosphate; 150 mM NaCl, pH 7.4), stained with trypan blue, to exclude non-viable cells, and counted with a hemocytometer. Cells in duplicate flasks were subjected to pulse labeling with [³H]thymidine (15 μCi/5 ml) (DuPont NEN, Boston, MA) for 1 h. At the end of the pulse labeling the medium was discarded and the cells were washed with PBS. Cells were extracted with 1.5 ml of 5% trichloroacetic acid (TCA) for 30 min at room temperature. TCA was discarded and the cell precipitate was washed with an additional 2 ml TCA. Precipitates were dissolved in 0.5 ml of 1 N NaOH which was then added to a scintillation vial containing 5 ml scintillation cocktail (Fisher Scientific, Springfield, NJ) and counted in a scintillation counter.

2.3. Determination of nitrite levels in medium

Nitrite concentrations were determined using the Griess reagent, essentially as described previously [22]. Aliquots of media (400 μl) were mixed with equal volumes of the Griess reagent (a solution containing 1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine in 2% phosphoric acid) and the optical density was determined at 540 nm. A standard curve was generated using sodium nitrite [22].

2.4. Determination of apoptosis

Apoptosis was detected by analyzing the presence of DNA ‘laddering’ on 1% agarose gels [23], as well as by flow cytometric determination of DNA content by staining with propidium iodide [24], and staining by propidium iodide of nuclei of unpermeabiized cells. Apoptosis was also determined in situ by Annexin V staining of the plasma membrane. To this end, rat vascular smooth muscle cells were seeded at 1 × 10⁴ to 2 × 10⁴ cells well⁻¹ in Nunc 16-well tissue culture slides (Nalge Nunc International, Naperville, IL) in 200 μl of DMEM/10% FBS supplemented with 1% penicillin–streptomycin (5000 U/ml/5000 μg/ml), 1% MEM non-essential amino acids (10 mM) and 1% MEM sodium pyruvate (100
mM), and cultured as described elsewhere [16,17]. After 18 h of incubation, the cells were stimulated with various nitric oxide donors: sodium nitroprusside (SNP) (Sigma, St. Louis, MO), and NOC-18, DETA/NO, (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-1,2-diolate (DETA NONOate) (Alexis Corporation, San Diego, CA). Cells were incubated in the presence of these donors at 37°C for the time indicated. Cells undergoing apoptosis were characterized by immunofluorescence staining with FITC-labeled Annexin V, as described in the Annexin apoptosis detection kit (R&D Systems, Minneapolis, MN). Following the incubation, fluorescent-labelled cells were examined using an inverted fluorescence microscope.

2.5. Northern blot analysis

RNA isolation and Northern blot analyses were performed as described previously [24]. The blots were probed with cDNA encoding either mouse p53 (generous gift of Dr. T. Jacks) or c-myc (generous gift of Dr. G. Sonenshein) or with actin cDNA as a control.

2.6. Determination of intracellular reduced glutathione concentration

VSM cells were treated for 24 h with either 1 mM sodium nitroprusside (SNP) or 10 µg ml⁻¹ lipopolysaccharide (LPS), with or without 0.5 mM glutathione monoethyl-ester (Sigma, St. Louis, MO). At the end of the incubation, the cells were washed twice with an excess of PBS and precipitated with 80% perchloric acid. Samples were centrifuged for 10 min at 1000 × g and supernatant collected. Supernatants were analyzed for reduced glutathione (GSH) using high performance chromatography with an electrochemical detector as described previously [25].

3. Results

3.1. Effect of an NO donor on DNA synthesis and apoptosis in vascular smooth muscle cells

We studied the effect of NO on DNA synthesis and cell growth in cultures of rat VSM cells. NO generated by sodium nitroprusside (SNP) caused a significant inhibition of DNA synthesis (Table 1). Proliferation of cell populations depends both on completion of full mitotic cell cycles and on rates of cell death. Fragmentation of genomic DNA has been well established as a clear criterion for programmed cell death [23]. As shown in Fig. 1, NO generated by SNP or by an NO-releasing compound which does not release cyanide, DETA NONOate, induced apoptosis in VSM cells, as indicated by DNA fragmentation. Well characterized responses to NO, such as inhibition of DNA synthesis and relaxation of smooth

Table 1

<table>
<thead>
<tr>
<th>Addition</th>
<th>Nitrite (µM)</th>
<th>[³H]Thymidine incorporation (dpm × 10³)</th>
<th>Cell number (× 10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>ND</td>
<td>6.8 ± 1.9</td>
<td>3.7 ± 0.6</td>
</tr>
<tr>
<td>SNP</td>
<td>19.7 ± 2.0</td>
<td>2.7 ± 0.6</td>
<td>1.4 ± 0.2</td>
</tr>
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VSM cells were cultured in the absence or presence of the NO donor SNP at a concentration of 1 mM for 20 h, after which the medium and cells were collected for different analyses as described under Section 2. The results represent averages of three experiments with an indicated standard deviation. ND, not detectable.

Fig. 1. Nitric oxide, but not cGMP, induces apoptosis in vascular smooth muscle cells. VSM cells were cultured for 24 h in the absence (lane 1) or presence of 0.2 mM 8-bromo-cGMP (lane 2), or in the presence of 1 mM SNP (lanes 3), or in the presence of 0.5 mM DETA NONOate (lane 4). DNA prepared from cells was fractionated on 1% agarose gel (10 µg/lane). A 1 kb DNA ladder (GIBCO–BRL, Gaithersburg, MD) was used as a marker (M).
muscle, are cGMP-mediated [5,26]. The cGMP analog, 8-bromo-cGMP, did not mimic the effect of NO on apoptosis in VSM cells (Fig. 1). We used two additional criteria for monitoring apoptosis: one involves flow cytometry analysis of the fraction of DNA stained by propidium iodide and which does
not correspond to diploid or higher ploidy sets of chromosomes; and the second is based on the ability of apoptotic cells to bind Annexin V due to changes in the cell membrane structure [27]. As shown in Fig. 2, flow cytometry analysis indicated that the fraction of cells undergoing apoptosis increased in proportion to the concentration of nitrite produced by SNP. In accordance, a similar fraction (about 15–30%) of the cells treated with DETA NONOate (Fig. 3) or SNP (not shown) displayed a clear binding to Annexin V as revealed by in situ staining, while control cells did not display a significant binding.

3.2. Effect of lipopolysaccharides on apoptosis in vascular smooth muscle cells

The production of NO from the guanidino nitrogen atom of L-arginine is mediated by the action of a group of enzymes known as NO synthases [5]. In endothelial cells, an isozyme is expressed constitu-

Fig. 3. In situ analysis of apoptosis in VSM cells treated with NO donors. VSM cells were incubated in the presence of 0.5 mM DETA NONOate (A,B) or in the absence of an NO-donor (C,D) for 24 h, after which they were stained with FITC-labeled Annexin V. Cells were viewed under phase (A,C) or fluorescent (B,D) microscope. Magnification ×250. Results are representative of three experiments. The arrows point to some of the cells undergoing apoptosis. Similar results to the one shown in panel D were obtained when cells were incubated with SNP (not shown). Other details are under Section 2.
Fig. 4. Effect of lipopolysacharides on apoptosis. VSM cells were incubated in the absence (lane 1), or presence of LPS 10 μg ml⁻¹ (lane 2), or LPS plus 5 mM L-NMA (lane 3), or LPS and 500 μM GSH monoethylester (lane 4), or 1 mM SNP and 500 μM GSH monoethylester (lane 5) for 24 h. DNA prepared from cells was fractionated 10 μg/lane on 1% agarose gel. A 1 kb DNA ladder GIBCO-BRL was used as a marker (M). In LPS-treated cells, the average level of nitrite determined in the medium (see Section 2) was 1 μM, while the level in non-treated cells was not detectable. The results are representative of three experiments.

3.3. Effect of sodium nitroprusside and lipopolysaccharides on the level of reduced glutathione in vascular smooth muscle cells

Since NO-induced apoptosis is cGMP independent, we tested the possibility that the effect of NO, produced by either SNP or LPS, was dependent on its effects on the levels of reduced glutathione (GSH). We focused on GSH since NO reacts with a variety of thiol groups, either on proteins or in the form of glutathione, to exert various biologic effects [29]. The level of NO available to react with thiol groups on proteins would be affected by GSH availability in the cells. As may be expected, LPS or SNP significantly decreased the level of GSH in VSM cells (Table 2). Addition of reduced glutathione (glutathione monoothylester, which enters cells freely [30] prevented this NO-induced reduction in intracellular GSH (Table 2). More interestingly, raising the concentration of reduced glutathione in the cells also prevented NO-induced apoptosis, as indicated by a complete lack of laddering of genomic DNA (Fig. 4).

3.4. The levels of p53 and c-myc in VSM cells exposed to high levels of NO

Induction of apoptosis in some cell types has been linked to high expression of the oncogene myc and in other lineages to the tumor suppressor gene p53 [14–16,31]. In VSM, c-myc is elevated in response to mitogens while it is down regulated in response to factors that induce growth arrest [32,33,26]. We, therefore, tested whether NO-induced apoptosis in VSM cells is associated with alterations in the levels of c-myc and/or p53 mRNAs. As shown in Fig. 4, within 8 h of treatment with LPS there was a significant increase in the level of p53 mRNA and a

Table 2

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<thead>
<tr>
<th>Addition</th>
<th>GSH (μmol/g protein)</th>
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<tr>
<td>None</td>
<td>9.69 ± 3.64 (11)</td>
</tr>
<tr>
<td>LPS</td>
<td>5.82 ± 1.89 (10)</td>
</tr>
<tr>
<td>SNP</td>
<td>5.91 ± 1.19 (10)</td>
</tr>
<tr>
<td>GSHe</td>
<td>19.67 ± 4.96 (6)</td>
</tr>
<tr>
<td>LPS + GSHe</td>
<td>8.97 ± 1.53 (3)</td>
</tr>
<tr>
<td>SNP + GSHe</td>
<td>9.19 ± 1.70 (3)</td>
</tr>
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</table>

VSM cells were cultured in the absence or presence of the NO donor SNP at a concentration of 1 mM or with LPS (10 μg ml⁻¹) for 24 h, in the absence or presence of 500 μM GSH monoethyl-ester (GSHe). Intracellular GSH was determined as described under Section 2. The results represent the average ± standard deviation for the number of experiments indicated in parentheses. The statistical significance between the treatment groups was determined with Students-test. When comparing the values in control and SNP-treated or control and LPS-treated cells, the differences were statistically significant (P < 0.001).
Figure 5. Lipopolysaccharide increases the level of p53 mRNA in VSM cells. VSM cells were incubated in the absence (lanes 1, 2) or presence of LPS (10 μg ml⁻¹) (lanes 3, 4) for 8 h (lanes 1, 3) or 24 h (lanes 2, 4). Northern blot analyses were performed on RNA isolated from cells incubated under the different conditions indicated. Blots, loaded with 10 μg RNA in each lane, were probed with either cDNA encoding mouse p53 or c-myc or with actin cDNA to confirm equal loading.

Figure 6. GSH monoethyl-ester abolishes NO-induced increase in p53. VSM cells were incubated in the absence (lane 1) or presence of LPS (10 μg ml⁻¹) (lane 2), or LPS plus 500 μM GSH monoethylester (lanes 3) for 24 h. Northern blot analyses were performed on RNA isolated from cells. Blots, loaded with 10 μg RNA in each lane, were probed with cDNA encoding mouse p53 or with actin cDNA (to confirm equal loading). GSH monoethylester added alone did not affect the baseline level (non-treated cells) of p53 (data not shown).

Fig. 5. Lipopolysaccharide increases the level of p53 mRNA in VSM cells. VSM cells were incubated in the absence lanes 1,2 or presence of LPS (10 μg ml⁻¹) (lanes 3,4) for 8 h (lanes 1,3) or 24 h (lanes 2,4). Northern blot analyses were performed on RNA isolated from cells incubated under the different conditions indicated. Blots, loaded with 10 μg RNA in each lane, were probed with either cDNA encoding mouse p53 or c-myc or with actin cDNA to confirm equal loading.

decrease in the level of myc mRNA. The alterations in the levels of these specific mRNAs were not transient, as concluded from analyzing cells cultured for longer incubation times (Fig. 4). It is of interest to note that a recent study has shown that apoptosis occurs in restenotic and atherosclerotic lesions of the blood vessel in association with detectable levels of p53 [34]. Increased concentrations of reduced glutathione in the cells also prevented the NO-induced increase in p53 (Fig. 5). It should be pointed out that the available antibodies to human p53 recognize mouse protein but not the rat p53, thus preventing us from following the level of p53 protein in our experiments. It is, however, reasonable to assume that conditions which result in equal levels of p53 mRNA (untreated cells and cells treated simultaneously with NO and GSHe) should lead to comparable levels of p53 protein. Since the level of p53 mRNA was increased in cells treated with NO but restored to control levels in the presence of GSH, and since GSH also inhibited NO-induced apoptosis, it is conceivable that the effect of NO on apoptosis is related to upregulation of p53 (Fig. 6).

4. Discussion

Inducible forms of NO synthase can be activated by interleukin(IL)-1β or lipopolysaccharide (LPS) in numerous tissues [27,5]. IL-1β or LPS-induced production of NO leads to elevation of intracellular cGMP [3,35], resulting in inhibition of cell proliferation [35]. In the current study, we have sought to determine whether the effect of nitric oxide on cell growth in VSM involves induction of programmed cell death. We found that NO generated by SNP or synthesized in VSM induced DNA fragmentation typical for apoptosis. It is of particular interest to note that in B lymphocytes NO inhibits apoptosis, also determined by DNA fragmentation [36], indicating that the nature of the effect of NO depends on lineage-specific signaling. In agreement with our results, Geng and colleagues [37] reported recently that IL-1β induces human VSM cells to produce nitrite and to undergo apoptosis, as indicated by DNA fragmentation. Our study, however, has been extended to explore the potential mechanisms by which NO induces programmed cell death.

Regulation of apoptosis has been extensively studied in the past few years. Apoptosis is inhibited in a variety of cell types by physiologic effectors, such as CD40 ligand, and by several viral genes. Inducers of apoptosis include members of the TNF family, TGF-β, growth factor withdrawal, and inducers of DNA damage [13]. In order to explore the mechanism by which apoptosis is initiated, the levels of different oncogenes and tumor suppressor genes were determined in cells treated with the above agents. Tumors that are deficient in p53 have enhanced resistance to
DNA damage and a delayed programmed cell death response [15]. Elevated c-myc expression in CHO cells or in fibroblasts with a block to cell proliferation induces apoptosis [31,38]. c-myc, however, also plays a central role in the proliferation of many cell types, including VSM cells. c-myc is induced in VSM cells exposed to mitogens or after balloon catheter arterial injury, and remains elevated throughout the cell cycle [39]. In accordance with this latter study, incubation of VSM cells with a cGMP analog results in downregulation of c-myc leading to arrest in cell growth [26]. These studies have led to the hypothesis that c-myc presents a ‘two signal’ model in which it can provide a signal leading to apoptosis or a signal leading to proliferation, depending on the cell type and cell cycle conditions. In the present study, we found that NO-induced apoptosis in VSM cells was accompanied by a decrease in the level of c-myc and a significant elevation of p53 mRNA.

Some responses to NO, such as inhibition of DNA synthesis, relaxation of VSM cells or inhibition of platelet aggregation, are cGMP-mediated [40]. Our study indicated that a cGMP analog did not mimic the effect of NO on apoptosis in VSM cells. NO effects that are independent of cGMP could be induced via reaction with thiol groups, as well as with oxygen, superoxide, or transition metals, yielding products that support additional nitrosative reactions, particularly S-nitrosothiol formation [5,29]. In the current study, we found that NO-induced apoptosis in VSM cells was associated with a significant decrease in the level of reduced glutathione (GSH) in the cells. Moreover, increasing levels of reduced glutathione in cells cultured with GSH monoethylester, prevented the NO-induced increase in p53 and protected against NO-induced apoptosis. These findings are consistent with several other reports on other systems. Petit and colleagues [41] have recently shown that glutathione protects against the antiproliferative effects of nitric oxide, but did not examine if some of the effect reflects an influence on the apoptotic process. Slater and colleagues [42] have demonstrated that thymocyte apoptosis is associated with a 40% decrease in intracellular GSH levels. In additions, it has been demonstrated that augmentation of cellular GSH with N-acetylcysteine attenuates apoptosis in U937 cells [43]. Furthermore, Kane and colleagues [44] have recently shown that expression of the proto-oncogene Bcl-2, a known inhibitor of apoptosis, is associated with an increase in intracellular GSH.

Our studies implicate GSH as having an essential role in protecting VSM cells from apoptosis. Our findings also demonstrate that high levels of GSH in the VSM cells prevent NO-induced increases in p53, a known stimulator of apoptosis. It is possible that GSH at high levels competes with other proteins for NO and, thus, reduces NO-induced modification of thiol groups on proteins, the later being potentially important for inducing apoptosis. For example, it has been shown that NO decreases the number of thiol groups in G proteins, resulting in direct nuclear translocation and activation of the transcription factor NF-kB [45]. Since NF-kB stimulates the production of p53 [46], it is conceivable that NO-induced upregulation of p53 is mediated via activation of NF-kB. The suggestion that NO induces apoptosis in VSM cells via p53 would be consistent with studies in other cell types in which apoptosis was attributed to induction of p53 [13,15]. Alternatively, since NO also alters the level of GSH in VSM cells, it is possible that GSH per se, by an as yet unidentified mechanism, prevents apoptosis, as suggested by Cossarizza and colleagues [47]. Our data, however, do not exclude the possibility that NO-mediated changes in the intracellular redox state are effecting the expression of other potential regulators of apoptosis in vascular smooth muscle, such as Fas protein [48]. Regardless of the nature of the end target of NO signaling (e.g., p53 or /and Fas), the effect on apoptosis seems to be mediated by changes in GSSG/GSH, as access of GSH prevents NO-induced programmed cell death.

The functional significance of apoptosis in VSM cells is probably critical in determining arterial structure, and remodelling in response to injury. Although luminal smooth muscle cells divide at high rate following arterial trauma, intimal thickening ultimately ceases [10]. These studies imply that cell death prevents complete occlusion of the artery. Our results provide a mechanism by which NO, either originating from endothel cells or produced in VSM cells themselves, could influence cell growth in the vasculature. We propose that availability of IL-1β in lesions [49], and thus increased levels of NO, should initiate a cGMP-dependent inhibition of DNA synthesis as well as a cGMP-independent activation of apoptosis, which can be prevented by increasing intracellular GSH.
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