Nitric Oxide Induces Oxidative Stress and Mediates Cytotoxicity to Human Cavernosal Cells in Culture

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ABSTRACT: Nitric oxide (NO) is a product of nitric oxide synthase (NOS) activity and is recognized as the main mediator of penile erection by induction of cavernosal smooth muscle relaxation. Although excessive NO can be generated via inducible NOS activation under certain inflammatory and noninflammatory conditions, for example, in response to TGF-β and γ-IFN (the proinflammatory cytokines), the effect of excessive NO produced as reactive nitrogen radical (NO·⁻) in the corpora cavernosa is not known. The present study was designed to evaluate whether the effect of NO·⁻ on human cavernosal cells in primary culture is via oxidative stress. Cell growth was monitored by DNA synthesis, and mitochondrial function was evaluated by adenosine triphosphate (ATP) production. Primary culture was initiated with explants from human corpora cavernosa, and the monolayer cavernosal cells (passage 2–3) were plated on 12-well tissue culture plates. At 70%–80% confluency, the cells were incubated with varying concentrations of sodium nitroprusside (SNP) for 16 hours. The cell growth (DNA synthesis) was monitored by measuring [^{3}H] thymidine incorporation, ATP levels (nanomoles per 10^4 cells) were measured by chemiluminescence assay using a luminometer, the total oxidative stress was monitored by measuring the levels of 8-iso PGF$_{2\alpha}$ (picograms per milliliter) by using an enzyme-linked immunosorbent assay kit, and NO production was monitored by accumulation of nitrite levels (micrometer per 10^4 cells). Human cavernosal smooth muscle cells (HCSMC) exposed to SNP (0 to 0.8 mM) exhibited a dose-dependent (two- to fivefold) decrease in DNA and ATP synthesis, accompanied by a two- to threefold increase in the levels of 8-iso PGF$_{2\alpha}$ and about an eightfold increase in nitrite accumulation. These findings suggest that the NO released by SNP (>0.8 mM) exhibited a significant cytotoxicity to HCSMC, mediated by increased oxidative stress to these cells.

Key words: Nitric oxide radical, ATP.

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Nitric oxide (NO) is the nonadrenergic, noncholinergic mediator of cavernosal smooth muscle relaxation responsible for penile erection (Burnett, 1995). In vivo and in vitro studies suggest that penile NO produced by NO synthase (NOS) diffuses into the smooth muscle cells, where activation of guanyl cyclase leads to relaxation of the corpora cavernosa (Rajfer et al, 1992; Leone et al, 1994). These physiological observations are further supported by histochemical demonstration of various isoforms of NOS in the cavernosal and penile nerve fibers (Burnett et al, 1993; Rajasekaran et al, 1998). Mammalian NO synthesis is mediated by at least 3 isoforms of NOS. Constitutive Ca^{2+}- and calmodulin-dependent NOS isoforms in the endothelium (eNOS) and peripheral neurons (nNOS) are associated with the formation of NO involved in signal transduction mechanisms (Griffith and Steehr, 1995). The third isoform, inducible NOS (iNOS), is Ca^{2+} and calmodulin independent and can be induced by bacterial lipopolysaccharide and inflammatory cytokines (Lyons et al, 1992).

Cavernosal smooth muscle is the primary cellular component of the human penis and the main target of NO during penile erection (Andersson and Wagner, 1995). Although human cavernosal cells in culture exhibit characteristic features of smooth muscle cells (Krall et al, 1988; Dahiya et al, 1993), it is unknown whether these cells can produce NO during in vitro propagation. Rat cavernosal cells express iNOS in a cell passage–dependent fashion, especially when exposed to inducing agents like bacterial lipopolysaccharide (LPS) or lymphokines (Hung et al, 1995). Recent studies from our laboratory also suggest that human cavernosal cells in culture may have the ability to synthesize their own NO when subjected to induction by proinflammatory agents (Rajasekaran et al, 1998). iNOS, which does not require calcium for activity, produces much higher levels of NO and for longer duration than nNOS or eNOS. This excessive NO·⁻ reacts more rapidly with superoxide and forms peroxynitrite (ONOO⁻), a more toxic radical (Radi et al, 1991).

It is possible that excessive NO·⁻ could be generated under certain inflammatory conditions in the corpora cavernosa (eg, Peyronie disease), leading to a variety of pathological effects in the penile architecture. Overproduction of NO·⁻ and NO·⁻-derived oxidants (peroxynitrite) has been shown to be highly cytotoxic (Radi et al, 1991). This

will inhibit a number of key regulatory enzymes and affect many metabolic pathways, such as the Krebs cycle, mitochondrial electron transport chain, and glycolytic pathway (Radi et al, 1994). In this study, we investigated the effect of sodium nitroprusside (SNP; as a source of excessive NO) on cellular adenosine triphosphate (ATP) levels, mitochondrial cytotoxicity, DNA biosynthesis, and the formation of 8-iso $PGF_{2\alpha}$ (as an indicator of oxidative stress) in human penile cavernosal smooth muscle cells in primary culture.

Materials and Methods

Human Cavernosal Cell Culture

Proper institutional review board approval was obtained, and cavernosal tissue explants (1–2 mm^3 in size) were obtained from patients undergoing penile prosthesis implantation. The fragments were washed aseptically and placed into culture flasks containing growth medium (Dulbecco minimum essential medium [DMEM] containing 20% fetal bovine serum [FBS]) and kept undisturbed for 4 days. The medium was exchanged every 4 days with fresh growth medium containing 10% FBS until the cultures became confluent. These primary cultures were characterized by specific immunohistochemical assays for α-actin and desmin expression to establish the presence of smooth muscle cells (Dahiya et al, 1993).

Study Design

Human cavernosal smooth muscle cells (HCSMC, 2 to 4 passages) were trypsinized at 80%–90% confluence and plated on either 24-well or 96-well culture plates. Cells were incubated with varying concentrations of SNP for up to 16 hours. Culture media was collected for nitrite and 8-iso $PGF_{2\alpha}$ assays. Cells were washed, counted, and checked for viability (by trypan blue dye exclusion test) and evaluated for mitochondrial respiration and DNA synthesis by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay and [³H]thymidine uptake, respectively. Mitochondrial function was evaluated by measuring total ATP.

Determination of ATP

Cells were plated in 24-well culture plates (10–20 × 10⁴ cells per well) and exposed to sodium nitroprusside (0–0.8 mM) for 16 hours. They were then trypsinized and pellet washed with cold PBS. Intracellular ATP levels were measured with a commercially available firefly luciferase bioluminescent assay kit (Labsystems, Helsinki, Finland). ATP was extracted from cells with Triton X-100 (0.1%) containing EDTA (4 mM) and assayed as follows: an aliquot (50 μL) was mixed with 110 μL of Tris-acetate buffer (0.1 M; pH 7.75) in a 96-well plate. ATP reagent (40 μL) containing firefly luciferase was dispensed using the luminometer pump. Chemiluminescence signal produced as a result of enzyme reaction catalyzed by firefly luciferase was measured using a Luminometer (Labsystems) and compared against standard ATP concentrations. The data was expressed as nanomoles of ATP generated per 10 000 cells (Armstrong et al, 1998).

Evaluation of Mitochondrial Activity

Mitochondrial activity as a test of cytotoxicity was evaluated by assessing MTT dye uptake. HCSMC were seeded at an initial density of 2 × 10⁴ cells per milliliter in 96-well plates and were grown to subconfluency in the 10% FBS-DMEM medium. The cells were washed in Hanks balanced salt solution (HBSS) and exposed to SNP (0–0.8 mM). After 16 hours of incubation, media was removed and then replaced with 100 μL of MTT (5 mg/mL in DMEM). After 2 hours, 150 μL of acidic isopropanol was added to each well, cells were lysed by repeated pipetting, and the absorbancy was read in an enzyme-linked immunosorbent assay (ELISA) plate reader at 570 nm wavelength (Ni and Hollander, 1996).

Evaluation of Cell Growth in Presence of NO

[³H] thymidine uptake in human cavernosal smooth muscle cells was evaluated as an index of DNA synthesis and cellular growth. Cells were plated at a density of 2–4 × 10⁴ cells per well in 24-well plates and grown in 10% FBS-DMEM for 2 days. Media was removed and supplemented with serum-free DMEM–0.5% albumin for 24 hours to reach the quiescent stage. At the end of this period, SNP (0–0.8 mM) was added to the wells and incubated for another 16 hours. During the last 6 hours of incubation, [³H] thymidine (0.5 μCi per well) was added in the same media. At the end of the incubation period, the supernatant was aspirated, the cells washed twice with cold PBS, and 0.5 mL of 10% ice-cold trichloracetic acid was added. The washings were transferred into glass tubes and pellet solubilized with 50 μL of 1% sodium dodecyl sulfate in 0.3 N NaOH. Radioactivity in the resulting solubilized fractions was quantified by scintillation counting (Wen et al, 1996).

Evaluation of Oxidative Stress

8-iso $PGF_{2\alpha}$ assay was used as marker of total oxidative stress (Morrow et al, 1990). The supernatants obtained after SNP incubation were quantified by a specific competitive enzyme immunoassay kit (Assay Designs, Ann Arbor, Mich) using an ELISA reader. These kits are very specific for 8-iso $PGF_{2\alpha}$, and cross reactivity for other prostanoid is approximately 1% to 12% (Armstrong et al, 1998).

Nitrite Measurement

Nitrite was determined in cell culture media as a stable end product of NO^- generated by the cells on incubation with SNP. These determinations used a spectrophotometric assay based on the Greiss reaction, measuring the absorbance at 550 nm (Grisham et al, 1996).

Statistical Analysis

The InStat statistics program (Graph PAD Software, San Diego, Calif) was used to compare the data obtained from the different treatments by 1-way analysis of variance (ANOVA), followed by nonparametric Newman-Keuls test. P values of $<.05$ were considered statistically significant.
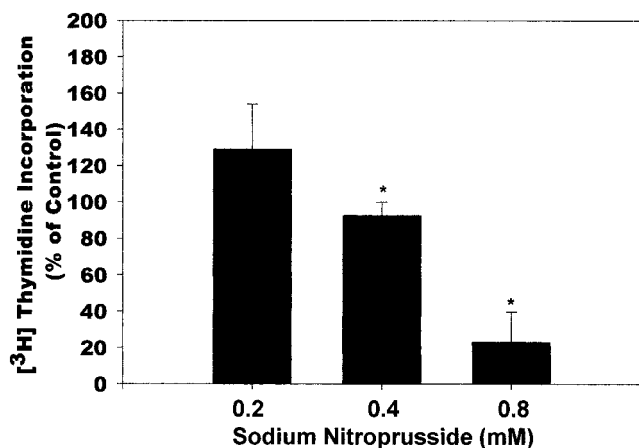
Figure 1. Effect of sodium nitroprusside (SNP) on DNA synthesis of human cavernosal smooth muscle cells. Cells were exposed to varying concentrations of SNP (0–0.8 mM) for 16 hours, and [3H] thymidine uptake was monitored as described under "Methods." Data represent mean ± SEM (n = 4). *$P < .05$ compared with control.
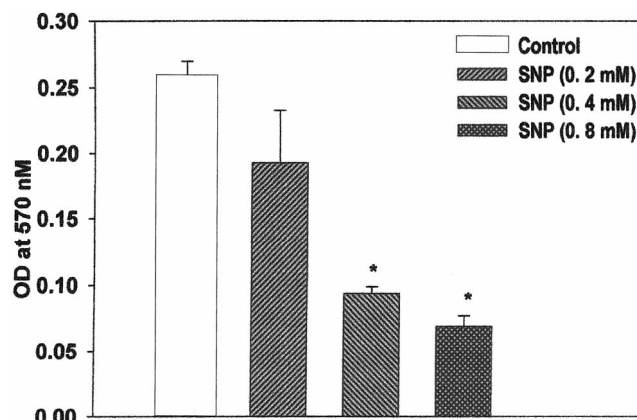

Figure 2. Effect of sodium nitroprusside (SNP) exposure on human cavernosal smooth muscle cell mitochondrial activity as measured by MTT assay. Cells were exposed to varying concentrations of SNP (0–0.8 mM) for 16 hours, and MTT dye uptake was determined as described under "Methods." Data represent mean ± SEM (n = 4). *$P < .05$ compared with control.

Results

Effect of SNP on HCSM Cell Mitochondrial Activity and Growth

SNP incubation induced a dose-dependent decrease in cell growth (thymidine uptake) and mitochondrial activity (MTT assay). DNA synthesis as monitored by [3H] thymidine uptake decreased marginally with 0.2 mM dose, whereas with the highest dose (0.8 mM), a significant (78%) decrease in cell growth was observed (Figure 1). SNP at the 0.2-mM dose level produced a 25% decrease in mitochondrial activity, whereas at the highest (0.8 mM) dose level, the activity decreased by 75% (Figure 2).

Effect of SNP on ATP and Nitrite Levels

ATP levels in HCSMC (nanomoles per 10^4 cells) decreased two- to threefold ($P < .05$) in a dose-dependent manner on exposure to SNP for 16 hours when compared with the case of controls. With 0.8 mM SNP, ATP levels were 77 ± 2.7 nM, compared with 198 ± 12 nM in control (Figure 3). SNP incubation resulted in a corresponding increase in NO·−, as monitored by nitrite levels (in micrometers per 10^4 cells) in the supernatants. Nitrite levels showed a dose-dependent three- to eightfold increase after incubation with SNP (Figure 4).

SNP-Induced Oxidative Stress Evaluation

Oxidative stress to cells from NO·− was monitored by specific isoprostanoid (8-iso $PGF_{2\alpha}$) assay. Exposure of cells to SNP (0.2–0.8 mM) produced a dose-dependent increase in the levels (in picograms per milliliter) of this isoprostanoid, which is a lipid hydroperoxide generated by noncyclooxygenase and/or lipoxygenase pathway in response to oxidative stress (Morrow et al, 1990). SNP at

the 0.2-mM dose level produced a twofold increase in 8-iso $PGF_{2\alpha}$, whereas at the highest dose (0.8 mM), the levels of this isoprostanoid reached a threefold increase (Figure 5).

Discussion

In the present study, exposure of human cavernosal cells to the NO·−-generating agent SNP produced a dose-de-
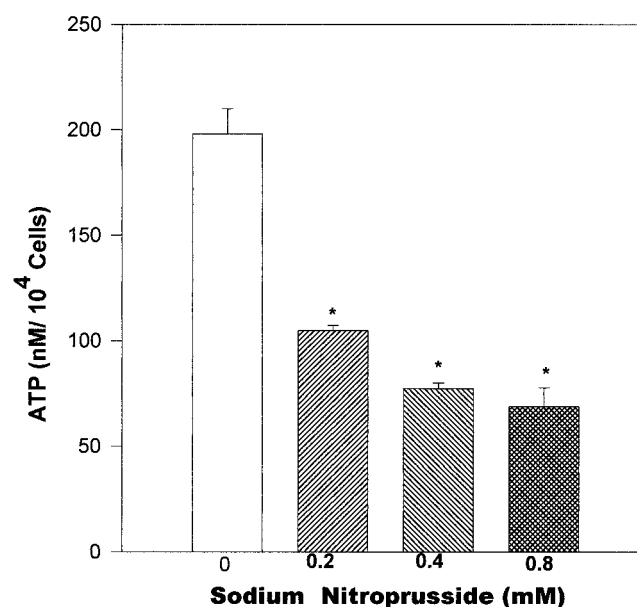

Figure 3. Effect of SNP on adenosine triphosphate (ATP) content of human cavernosal smooth muscle cells. Cells were exposed to varying concentrations of SNP (0–0.8 mM) for 16 hours, and intracellular ATP levels were determined as described under "Methods." Data represent mean ± SEM (n = 4). *$P < .05$ compared with control.
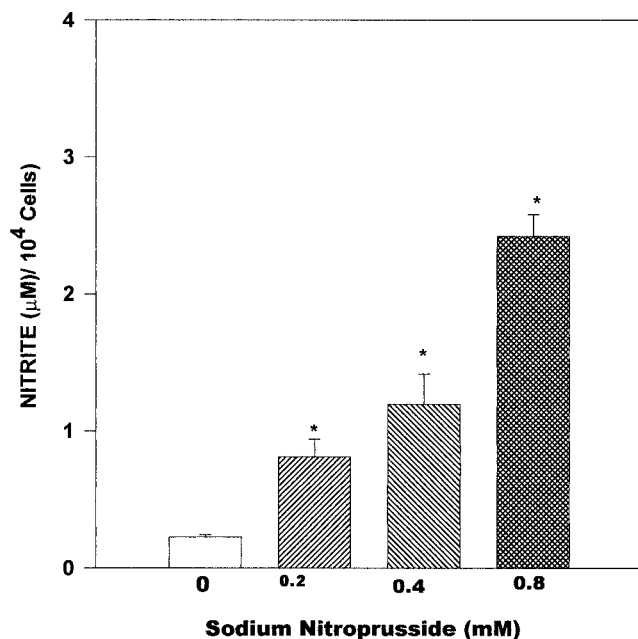
Figure 4. Effect of SNP exposure on nitrite accumulation of human cavernosal smooth muscle cell culture. Cells were exposed to varying concentrations of SNP (0–0.8 mM) for 16 hours, and nitrite accumulation in the medium was determined as described under "Methods." Data represent mean $\pm$ SEM (n = 4). *$P < .05$ compared with control.

Figure 5. Effect of SNP exposure on 8-iso $PGF_{2\alpha}$ levels of human cavernosal smooth muscle cell culture. Cells were exposed to varying concentrations of SNP (0–0.8 mM) for 16 hours, and 8-iso $PGF_{2\alpha}$ accumulation in the medium was determined as described under "Methods." Data represent mean $\pm$ SEM (n = 4). *$P < .05$ compared with control.

pendent depletion of intracellular ATP, MTT reactivity, and [³H]thymidine uptake. These data suggest the inhibition of mitochondrial function and DNA biosynthesis, respectively. A dose-dependent accumulation of 8-iso $PGF_{2\alpha}$ and nitrite in the media implies increased oxidative stress and that these metabolic effects are mediated by NO.

NO is the central mediator of penile erection, and altered NO synthesis or action has been associated with erectile impairment during conditions like aging (Garban et al, 1995) and diabetes (Vernet et al, 1995). Conversely, an excess of NO production could lead to pathological consequences in the penile tissue or cavernosal smooth muscle cells, which are the main target of $NO^{·-}$ action. It is reasonable to assume that cavernosal cells may be exposed to excessive $NO^{·-}$ by iNOS induction in conditions such as Peyronie disease, trauma, and priapism. NO is capable of interacting with a number of cellular targets, including heme and nonheme iron, thiols, oxygen, and superoxide anion (Radi et al, 1991). Reaction with these targets can result in either physiological effects, such as the activation of guanylate cyclase, or pathological effects, such as the production of peroxynitrite (Wink et al, 1998). Many of the cytotoxic effects of $NO^{·-}$ are related to the production of peroxynitrite because of its rapid reaction with superoxide radicals. Many compounds and antioxidants will scavenge superoxide, NO, and peroxynitrile radicals. We did not evaluate peroxynitrite produc-
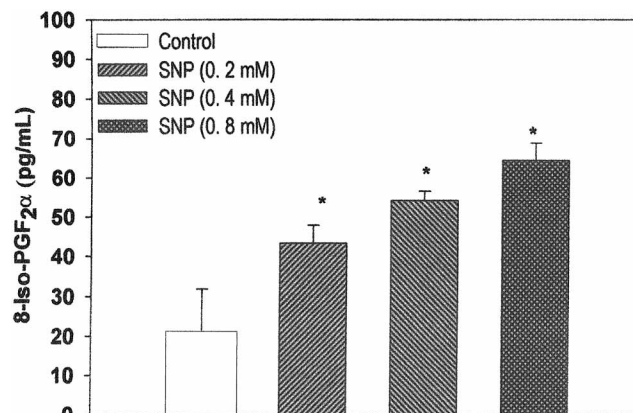
tion in this study, but measurement of nitrite-nitrate correlated with $NO^{·-}$ production from SNP in a dose-dependent manner (Figure 4). It is important to know that the balance between physiological regulation and pathological effect is dependent upon the relative concentrations of free radicals, the amount of biological antioxidants (like SOD, glutathione, or vitamin E) scavenging these radicals, and the type of reactive biological targets. Any agent that will scavenge peroxynitrite at the time it is bound to NOS, for example under low cellular arginine concentrations, will be highly effective in its cytoprotective role (Cuzzocrea et al, 1998). Further studies are required to explore this mode of cellular protection against $NO^{·-}$-induced cytotoxicity to cavernosal cells.

Production of NO in the penis during erection has been hypothesized to originate from the endothelium and the nerves innervating the corpus cavernosum. Later studies using specific antibodies demonstrated the presence of an endothelial type of NOS in the rat penis (Dail et al, 1995), and the subsequent immunoblot studies confirmed the presence of this 140-kd protein in the rat penile cytosol as well as in the particulate fractions (Penson et al, 1996). The type of NOS isoforms in the smooth muscle component of the penis and its role in the erectile mechanisms are not well understood. Rat smooth muscle cells can express iNOS when induced by bacterial lipopolysaccharide and recombinant γ-interferon (Hung et al, 1995). Our recent studies demonstrated that HCSMC in culture express both endothelial and cytokine-inducible isoforms of NOS, with substantial similarity at the level of mRNA to the human endothelial and hepatocyte enzymes (Rajasekaran et al, 1998).

Incubation of monolayer cells with SNP significantly reduced intracellular ATP levels. The effect of $NO^{·-}$ donor on ATP levels was concentration dependent. Our ob-

servations that exposure to $NO^{\cdot-}$ donor leads to depletion of ATP is consistent with the known ability of $NO^{\cdot-}$ to inhibit a number of enzymes involved in metabolic pathways leading to ATP synthesis. These enzymes include aconitase in the Krebs cycle, NADH-ubiquinone oxidoreductase and succinate-ubiquinone oxidoreductase of the mitochondrial electron transport chain, and glyceraldehyde-3-phosphate dehydrogenase in the glycolytic pathway (Bolanos et al, 1994). These effects have been observed in a variety of cell types, including hepatocytes (Niknahad and O'Brien, 1996), ovarian cells (Ellman et al, 1993), and macrophages (Messmer and Brune, 1996).

In addition to its effect on intracellular ATP stores, $NO^{\cdot-}$ also has been noted to impair proliferation of DNA synthesis and mitochondrial respiration (Radi et al, 1994). These metabolic effects were accompanied by elevated levels of 8-iso $PGF_{2\alpha}$, a specific marker of oxidative stress. Increased generation of isoprostanoid, particularly 8-iso $PGF_{2\alpha}$, has been demonstrated in vivo in several rat models of oxidant-induced injury (Nanji et al, 1994), in smokers (Mohler et al, unpublished data), and in hyperglycemic conditions (Natarajan et al, 1996). In human pulmonary organ culture, stimulation of cells with a combination of LPS, tumor necrosis factor-α, and Interferon-γ stimulated the release of 8-iso $PGF_{2\alpha}$, which was attenuated by the NO synthase inhibitor L-NAME (Jourdan et al, 1997). Our observations that the exposure of cells to NO donor results in accumulation of this metabolite suggest the involvement of oxidative stress in these cytotoxic actions of NO.

In conclusion, we have demonstrated that human cavernosal cell culture is a viable model for the evaluation of metabolic actions of $NO^{\cdot-}$. The evidence presented in this report on the depletion of energy stores and inhibition of DNA synthesis by SNP suggest the potential toxicity of $NO^{\cdot-}$ in disease processes associated with excess reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation. The question of whether these concentrations could be achieved in vivo under these pathological conditions, especially in Peyronie's disease, needs to be addressed.

Acknowledgments

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