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Nitric Oxide Promotes Resistance to Tumor Suppression by CTLs

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Many human tumors express inducible NO synthetase (NOS2), but the roles of NO in tumor development are not fully elucidated. An important step during tumor development is the acquisition of apoptosis resistance. We investigated the dose-dependent effects of endogenously produced NO on apoptosis using ecdysone-inducible NOS2 cell lines. Our results show that short-term NOS2 expression enhances CD95-mediated apoptosis and T cell cytotoxicity dose dependently. Furthermore, we could show that during chronic exposure to NO, besides the primary cytotoxic NO effect, there is selection of cell clones resistant to NO that show cross-resistance to CD95-induced apoptosis and the killing by CTLs. We propose that NO production could initially act as an autocrine suicide or paracrine killing mechanism in cells undergoing malignant transformation. However, once failed, the outcome is fatal. NO promotes tumor formation by enhancing the selection of cells that can evade immune attack by acquiring apoptosis resistance. The Journal of Immunology, 2006, 176: 3923–3930.

Nitric oxide plays key roles in physiological as well as pathological processes (1–3). NO functions as an intracellular and intercellular signaling molecule shaping the immune response (4). During inflammation, NO produced from the inducible NO synthetase (NOS2) in response to proinflammatory cytokines is critical for short-term defense against infection and tumor cells (5). But during longer episodes of inflammation, NO is capable of causing tissue damage and can be genotoxic and mutagenic (6–8). Extensive evidence indicates that tissues undergoing chronic inflammation have a significantly higher risk for cancer (9). Furthermore, aberrant production of NO by induction of NOS2 can be acquired by tumors. Human tumors such as breast cancer, melanoma, bladder cancer, hepatocellular carcinoma, and colorectal cancer often express high levels of NOS2 (10–13). The underlying roles that NO plays in tumor-host cell interactions are complex and may be dependent on the context and source of NO. For example, recent data using a genetic strategy in mice has provided evidence that NO can suppress tumorigenesis as p53−/−NOS2−/− mice developed tumors more rapidly than the corresponding wild-type mice (14). Furthermore, antitumor activity through NO production has been attributed to host macrophages (15). In contrast, significant experimental and clinical evidence suggests that tumor-derived NO is conducive to tumor progression and is detrimental to the host (16–19).

One of the major apoptosis signaling pathways is the CD95 receptor/ligand system. Alterations in the control of apoptosis mediated through the CD95 system contribute to the pathogenesis of disorders such as cancer, autoimmunity, AIDS, and liver diseases (20–26). Acquiring reduced sensitivity to CD95-mediated apoptosis is common in cancer cells and may provide them with critical survival advantages ultimately promoting malignancy (27–30). Furthermore, the selection for resistance to apoptosis-inducing signals may hinder elimination of tumors through immune surveillance. Tumor escape from immune surveillance has been hypothesized to result from the inability of the immune system to react to the tumor. CTLs have been consistently implicated in tumor surveillance and regression. One system which plays an effector role in immune surveillance is CD95-mediated apoptosis of tumor cells by infiltrating T lymphocytes. Thus, resistance to CD95-mediated apoptosis signals can conceivably lead to escape of tumor cells from immune surveillance (31–36).

In many studies, cells are exposed to NO through the use of donor drugs. The methods and substances used to deliver NO can significantly affect responses to NO, resulting in conflicting reports with respect to cytotoxicity and apoptosis. The total doses can be very high and rates of exposure can be well above physiological levels. Furthermore, the donor drugs themselves and their reaction by-products may affect cellular responses (37). Cell culture experiments with stable cell lines constitutively expressing NO have the disadvantage that selection occurs under constant NOS2 production. The possibility cannot be excluded that selection pressure is in part due to coping with constant NO exposure.

The molecular mechanisms underlying NO cytotoxicity are not completely unraveled. NO toxicity is generally thought to be mediated by mitochondria dysfunction, the DNA damage-p53 pathway (6, 38), and, more recently, endoplasmic reticulum (ER)3 dysfunction (39–42). Recent work has identified signals derived from the ER during states of stress which can induce apoptosis. In mice,

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3 Abbreviation used in this paper: ER, endoplasmic reticulum; CHOP, C/EBP-homologous protein; GADD153, growth arrest and DNA damage-inducible gene 153; BiP, Ig-binding protein.
high levels of ER stress can activate caspase-12, an ER-associated death effector caspase, which cleaves the downstream caspses such as caspase-9-inducing apoptosis (43). In humans, a functional caspase-12 protein is not produced, because the gene is interrupted by a frame shift mutation resulting in a premature stop codon (44). The gene in humans with the highest homology to rodent caspase-12 is caspase-4. Human caspase-4 is localized to the ER membrane and is cleaved when cells are treated with ER stress-inducing reagents (45). Thus, caspase-4 can function as an ER stress-specific caspase in humans.

In this study, we examined the effects of endogenously produced NO on the CD95 system, on the action of CTLs, and of long-term NO exposure in cells with edysone-regulatable NOS2 expression. Our results demonstrate that the primary effect of endogenously produced NO is to enhance the sensitivity for CD95-induced apoptosis and the killing by CTLs. We could show that persistent exposure to NO induces apoptosis without additional stimuli and selects for cells with reduced sensitivity to NO and apoptosis. We report that NO-induced apoptosis is mediated through an ER stress mechanism, involving the induction of C/EBP-homologous protein (CHOP)/growth arrest and DNA damage-inducible gene 153 (GADD153), the ER chaperone Ig-binding protein (BiP), and caspase-4. Collectively, our findings suggest that NO production in tumor cells is initially detrimental for their survival but promotes selection of variant apoptosis-resistant cells favoring tumorigenic growth.

Materials and Methods

Chemicals

The following materials were purchased from commercial sources: anti-NOS2 Ab, anti-caspase-3, -8, and -9 Ab (Apotech); anti-BiP Ab (Affinity Bioreagents); anti-CHOP Ab (Santa Cruz Biotechnology); anti-α-actin Ab (Biocarta); anti-rabbit, anti-mouse, and anti-goat secondary Abs, alkaline phosphatase-conjugated (Sigma-Aldrich); propidium iodide (Sigma-Aldrich); JC-1 (Molecular Probes); Griess assay reagent (Alexis Biochemicals); Cell Titer Glo cell viability assay kit (Promega); Lipofectamine 2000 reagent (Invitrogen Life Technologies); CDP-star Western blot detection system (Applied Biosystems).

Cell culture and cloning

EcR293 cells (human embryonic kidney (HEK) 293, stably transfected with pVgRXR/zeocin regulator vector), were grown in DMEM supplemented with 10% FCS, 5% penicillin and streptomycin, 5% l-glutamine, 5% HEPES buffer at 37°C in a humidified incubator with 5% CO2. The cDNA of NOS2 (inducible NO synthase) containing the entire open reading frame, and partial 5’ and 3’ untranslated sequences of NOS2 were cloned into an edysone-inducible mammalian expression vector, pLND(Sp1)/neomycin (Invitrogen Life Technologies). EcR293 cells were seeded in 6-well plates at 50% confluence, and transfected with pLND-NOS2 plasmid by Lipofectamine 2000 reagent according to the manual. The stable transfected cells were selected in medium containing both G418 (400 µg/ml) and zeocin (400 µg/ml). The clones growing up after ~4 wk of selection were picked up and further analyzed.

Western blot analysis

EcR293-NOS2 clones were seeded in 6-well plates, induced by ponasterone A. After a 48-h incubation, the cells were washed twice with PBS and lysed in 1% Nonidet P-40 solution. For Western blot, 40 µg of proteins were loaded on a 8% SDS-PAGE gel, separated, and transferred to a polyvinylidene difluoride membrane. The proteins were detected by indirect immunofluorescence and visualized by the CDP-star detection system.

Apoptosis analysis

EcR293-NOS2 cells were seeded in 12-well plates at 60% confluence, and the cells were induced for 24 h with ponasterone A. For apoptosis induction, the cells were treated with 300 ng/ml anti-APO-1 Ab for 24 h. DNA fluorescence was measured by FACS analysis after fixation with cold 100% ethanol and staining by propidium iodide according to a published method (46). Apoptotic sub-G1 cell nuclei were quantified using a FACSCalibur cytometer.

Griess assay

EcR293-NOS2 clones were seeded in 6-well plates, induced by ponasterone A at 0, 0.5, 1, 3, 10 µM concentrations for 48 h. Medium was collected for nitrite determination and the cells were harvested for normalizing each sample to total protein concentration. Griess assay was performed according to manufacturer’s protocol. Nitrite concentrations were determined at an OD595 by comparison with standard solutions of sodium nitrite prepared in the same culture media and presented in correlation to protein content.

Caspace activity analysis

The EcR293-NOS2 cells were seeded in 6-well plates, induced by 5 µM ponasterone A for 24 h and treated with 300 ng/ml anti-APO-1. After 0, 3, 6, 12, 24, 36 h treatment (or until 120 h for caspase-4 activation assay during ER stress), the cells were harvested in 1× PBS/1% Nonidet P-40 solution, and centrifuged at 13,000 × g for 5 min at 4°C. Protein concentration of the resulting supernatant was determined by using the Micro BCA protein assay kit (Pierce) protein assay kit. Supernatant containing, respectively, 10, 40, 50, and 50 µg of proteins was diluted with assay buffer and incubated for 2 h at 37°C with 500 µM caspase-3, -4, -8, or -9 substrate (Ac-DEVD-ACF, Ac-LEVD-ACF, Ac-IEHD-ACF, Ac-LEHD-ACF), respectively. Cleavage of the substrate was monitored at 405 nm and expressed in relative proteolytic activity.

Mitochondrial membrane potential analysis

Cells were seeded in 6-well plates, induced by ponasterone A for 24 h and treated with anti-APO-1 (300 ng/ml) for another 24 h. Then the cells were harvested and stained with JC-1 for 1 h at 37°C. After washing with PBS, the cells were resuspended and stained with JC-1. Red fluorescence of mitochondrial JC-1 aggregates was measured immediately by FACS analysis.

Cytotoxic T cell killing

An alloreactive A2.1-specific polyclonal CTL line (CD8 Allo-A2) was established as reported (47). An A2.1-restricted CTL clone specific for the Flu M1 58–66 peptide (CD8 × A2K(Flu)) has been described previously (48). CTLs were cocultured with 51Cr-labeled EcR293-NOS2 cells at the indicated E:T ratios. 51Cr release was measured after 4.5 h. The peptide specificity of the CD8 × A2K(Flu)M1 CTLs was controlled by their failure to lyse target cells loaded with an irrelevant peptide.

Viability measurement

Cell viability was determined by the CellTiter-Glo viability assay kit (Promega). For this purpose, the cells were seeded in 96-well plates and treated as described. After culturing for the indicated times, CellTiter-Glo reagent was added as recommended by the manufacturer. Luminescence was determined in a luminescence ELISA reader (Tecan).

Immunofluorescence

The cells were seeded in an 8-well slide chamber with coverslip bottoms (BD Biosciences) and induced with 5 mM ponasterone A for 48 h. After washing with PBS, the cells were fixed in 4% paraformaldehyde and stained for NOS2 or CHOP. Cy3-labeled anti-rabbit IgG (Dianova) was used as secondary Ab. Hoechst 33342 (Molecular Probes) was used to stain nuclei. The cells were imaged directly in the chambers using a Zeiss LSM 510 UV laser scanning microscope.

Selection of NO-resistant clones

EcR293-NOS2 no. 33 cells were seeded in 6-well plates at ~5–10% confluence and cultured in selection medium (containing 300 µg/ml G418, 300 µg/ml zeocin, and 10 µM ponasterone A). The medium was changed every 2 days. After ~3 mo of continuous selection, the clones were analyzed.

Results

Construction and regulatable expression of NOS2 in HEK-293 cells

The full-length human NOS2 gene was cloned into pLND/neomycin, which contains the edysone-responsive element sequences and the neomycin-resistance gene. The resulting vector, pLND-NOS2, was transfected in EcR293 cells which have stably integrated pVgRXR vector carrying both the retinoid X receptor and
the ecdysone receptor and a zeocin gene. Stable transfectants were selected which showed NOS2 expression in response to ponasterone A, an ecdysone analog, in a dosage-dependent manner (Fig. 1A). Immunofluorescent staining of EcR293-NOS2 clone no. 33 for NOS2 was consistent with Western blot results. NOS2 expression was detected in induced cultures localized in punctate structures (Fig. 1B). We monitored ecdysone-inducible NO production by Griess assay (Fig. 1C). These data demonstrate that EcR293-NOS2 cells produce NO in a regulatable and dose-dependent manner in response to ponasterone A treatment. NO levels correlate with NOS2 protein levels as characterized by Western blot and immunofluorescence analysis. A time course for induction of NOS2 after treatment with ponasterone A revealed maximal expression levels between 12 and 48 h, however, continuous expression of NOS2 could be detected throughout the time course of the experiment (Fig. 1D).

**Effect of NO on CD95-mediated apoptosis**

The effect of endogenously produced NO on CD95-mediated apoptosis was examined in EcR293-NOS2 cell clones. Fig. 2A shows CD95 apoptosis induction monitored by the amount of cells with a sub-G1 DNA content in the absence or presence of ponasterone A. An immunofluorescent staining of EcR293-NOS2 clone no. 33 was induced. These data demonstrate that EcR293-NOS2 cells produce NO in a regulatable and dose-dependent manner in response to ponasterone A treatment. NO levels correlate with NOS2 protein levels as characterized by Western blot and immunofluorescence analysis. A time course for induction of NOS2 after treatment with ponasterone A revealed maximal expression levels between 12 and 48 h, however, continuous expression of NOS2 could be detected throughout the time course of the experiment (Fig. 1D).

**Sensitivity of EcR293-NOS2 cells to CTL-induced apoptosis**

The CD95/CD95L system (APO-1/Fas) mediates perforin-independent cytotoxic T cell killing of tumor cells. To test whether cytolytic effector functions of CTLs are influenced by NO, we performed coculture experiments with allogenic or peptide-specific CTL (CD8 Allo A2 and CD8 × A2kbFluM1) and EcR293-NOS2 cells as targets. We found a substantial increase in the cytotoxic action of T cells in EcR293-NOS2 targets when NOS2 was expressed by induction with ponasterone A (Fig. 2C). This effect was dependent on the doses of ponasterone A (Fig. 2C) indicating that increasing NO levels in targets correspondingly increased their susceptibility for the killing by CTLs. The NO-producing target cells pulsed with an irrelevant peptide were not lysed during the time course of the coculture experiments excluding the possibility of autocrine suicide (Fig. 2C). Taken together, our results demonstrate that NO increases the sensitivity of EcR293-NOS2 cells to apoptosis induced by CD95 stimulation and the cytolytic action of CTLs.

**Apoptosis signaling**

To investigate whether the increased sensitivity to CD95-induced apoptosis is possibly due to increased CD95R expression, we measured CD95 surface expression by immunofluorescence staining and FACS analysis. No changes in CD95 levels were detected between the induced and uninduced cells (data not shown). We further tested the effect of NOS2 induction on caspases activated during CD95 apoptosis in cells uninduced and induced for production of NO. As shown in Fig. 3A, activation of caspases-3, -8, and -9 after CD95 stimulation was enhanced in cells when NOS2 was induced. These results indicate that NO can increase the CD95 apoptosis sensitivity in EcR293-NOS2 cells. Mitochondria act as a central integrator of the apoptotic response. Using JC-1, we analyzed the loss of mitochondrial membrane potential after expression of NOS2 and activation of the CD95 apoptotic pathway. Qualitative changes in the mitochondria membrane potential induced by exposure to NO and treatment with agonistic CD95 Abs (anti-APO-1) were observed using laser scan microscopy (data not shown). Aggregates of JC-1 in normal cells appeared red in the mitochondria, whereas damaged cells with mitochondrial membrane potential loss appeared with decreasing red due to loss of JC-1 aggregates. Quantitative analysis by flow cytometry revealed that short-term NO induction sensitized cells to mitochondria membrane potential loss during CD95-induced apoptosis in a dose-dependent manner (Fig. 3B).

**NO induces apoptosis**

We examined the effects of long-term exposure to NO in EcR293-NOS2 cells. Fig. 4A shows growth curves of EcR293 cells in the...
presence or absence of 5 μM ponasterone A. There was no apparent differences in the cell numbers of the control EcR293 cells treated with or without ponasterone A. However, induction of NOS2 in EcR293-NOS2 cells led to drastic reduction in cell number compared with cultures left untreated (Fig. 4B). To ensure that the growth retardation is dependent on the expression level of NOS2, we treated EcR293 and EcR293-NOS2 with various doses of ponasterone A for 24 h and then measured the cell viability. Compared with untreated controls, ponasterone A treatment did not influence the viability of the EcR293 cells, whereas in EcR293-NOS2 cells a dose-dependent reduction of the cell viability reaching 50% reduction with 10 μM ponasterone A was observed (Fig. 4C). Thus, the striking growth reduction was due to the effect of NO production.

To explore the possible mechanisms underlying the induction of apoptosis in cells exposed to longer periods of NO, NOS2-expressing EcR293 cells were treated with thapsigargin, an ER-stress inducer. The cytotoxic effects of thapsigargin increased with higher levels of NOS2 suggesting that NO enhanced the sensitivity of cells to toxic stimuli including ER stress (Fig. 5A). Expression of BiP, CHOP/GADD153, both induced during ER stress, and caspase-4, mediating ER-derived apoptosis signals, was analyzed. Procaspase-9, the mitochondrial caspase, that is recruited to the apoptosome where it is activated and released, was investigated. EcR293-NOS2 cells were treated with increasing concentrations of ponasterone A to induce NOS2 expression and then Western blot analysis was performed for the expression level of the ER chaperone BiP (glucose-regulated protein 78). In untreated cells, low levels of BiP were detected. As the time of NO exposure substantially over uninduced controls after 8 days of culture. These results indicate that endogenous NO reduces the cell number by inducing apoptosis in the cultures.

ER and mitochondrial stress after NO induction

To explore the possible mechanisms underlying the induction of apoptosis in cells exposed to longer periods of NO, NOS2-expressing EcR293 cells were treated with thapsigargin, an ER-stress inducer. The cytotoxic effects of thapsigargin increased with higher levels of NOS2 suggesting that NO enhanced the sensitivity of cells to toxic stimuli including ER stress (Fig. 5A). Expression of BiP, CHOP/GADD153, both induced during ER stress, and caspase-4, mediating ER-derived apoptosis signals, was analyzed. Procaspase-9, the mitochondrial caspase, that is recruited to the apoptosome where it is activated and released, was also investigated. EcR293-NOS2 cells were treated with increasing concentrations of ponasterone A to induce NOS2 expression and then Western blot analysis was performed for the expression level of the ER chaperone BiP (glucose-regulated protein 78). In untreated cells, low levels of BiP were detected. As the time of NO exposure substantially over uninduced controls after 8 days of culture. These results indicate that endogenous NO reduces the cell number by inducing apoptosis in the cultures.

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increased, the amount of BiP increased (Fig. 5B) indicating that NO stress induced BiP expression. The expression of the transcription factor CHOP/GADD153, which is induced during ER stress, was analyzed by immunostaining and Western blot. Compared with uninduced cells, the expression of CHOP/GADD153 was increased and was localized primarily in the nucleus under induction of NOS2 by ponasterone A (Fig. 5C). Caspase-9 expression was monitored by immunoblotting during 72 h of continuous NO exposure. Caspase-4 activity, measured by caspase assay was observed after 48–72 h of NO exposure suggesting that NO induced ER stress leading to activation of caspase-4 (Fig. 5D). Similarly, the activated form of caspase-9 increased during these cultivation conditions (Fig. 5D). Long-term exposure to NO resulted in extensive mitochondrial membrane potential loss without additional apoptosis stimulation (Fig. 5E). Together, these results show that endogenously produced NO activates ER and mitochondrial stress pathways which may play a direct or indirect role in triggering apoptosis.

**Continuous NO stress selects for NO- and apoptosis-resistant cells**

If long-term NO exposure induces apoptosis, then all NOS2-expressing cells should be eliminated or counteract this death stimuli by acquiring apoptosis resistance. To test this hypothesis, we cultured EcR293-NOS2 cells in the presence of 10 μM ponasterone A to continuously produce NO. We observed significant cell death in these cultures but cell colonies grew which were selected for further cultivation. After 3 mo of culturing in the presence of 10 μM ponasterone A, the cell clones were analyzed for NO production by Griess assay. All the cell clones which survived cultivation in the presence of 10 μM ponasterone A also produced NO (Fig. 6A). These results demonstrate that the survival of the colonies was due to NO resistance and not merely to loss of ponasterone A-inducible NO production. To assess the effects of the NO-resistant clones on apoptosis, cells were treated with agonistic CD95 Abs and apoptosis was analyzed by quantification of sub-G₁ cells. Interestingly, we found that the NO-resistant cell lines were also cross-resistant to CD95-induced apoptosis (Fig. 6B). Coculture experiments with different peptide-specific CTLs demonstrate an acquired resistance to CTL killing (Fig. 6C and D). These results support the idea that chronic exposure to NO selects cells with reduced sensitivity to apoptosis.
Discussion
NO is associated with tumor development and progression (2, 6, 7, 17–19). We have analyzed the cellular mechanisms by which endogenous NO exposure in tumor cells can modulate apoptosis sensitivity, giving particular attention to apoptosis mediated by the CD95 system. To this end, we constructed an NOS2 expression vector driven by an ecdysone-inducible promoter; the vector was then introduced into EcR293 cells. Ecdysone-dependent NOS2 expression and NO production was detectable in a number of cell clones. Using this cell culture model, we tested short- and long-term effects of endogenous NO exposure on CD95-mediated apoptosis. Our results demonstrate a dramatic enhancement of CD95-mediated apoptosis during acute episodes, with increased caspase activity and mitochondrial membrane depolarization. Furthermore, in coculture experiments, we show that the cytolytic action of T cells against NO-producing tumor cells is enhanced. In summary, short-term NO exposure should reduce tumor formation. In line with this observation, continuous intermediate-term exposure to NO initially reduced cell numbers and induced apoptosis. However, finally, we observed a selection for cells that were resistant to both NO and apoptosis after chronic NOS2 expression.

Diseases associated with chronic inflammation predispose individuals to cancer in the affected tissues. NO has attracted considerable attention as a possible link between inflammation and tumorigenesis for several reasons. First, due to its free radical nature, NO is capable of reacting at different points in the cell by modifying proteins and DNA. Second, NO is produced during inflammation by induction of NOS2. Third, NOS2 expression is observed in many tumors and is continued to be expressed in advanced tumors suggesting that it has a positive role for the tumor (49). Moreover, there is extensive experimental evidence to support a role for NO in accelerating tumor development but the cellular and molecular mechanisms by which NO promotes tumor formation and growth are not fully understood. One of the proposed mechanisms linking NO to carcinogenesis is the ability of NO to act as an endogenous mutagen and to modify intracellular signaling. For
example, recent reports have demonstrated an important role for p53 as modulator of genotoxicity and mutagenesis during NO-induced oxidative DNA damage (6, 7, 50, 51). Furthermore, the accumulation of DNA lesions during NO stress may be in part attributed to the inhibition of DNA repair enzymes by NO (52). Thus, continuous genomic insults due to NO production may have negative effects on genome stability promoting tumor progression.

However, short-term cytotoxic effects of NO have been clearly documented; the possible targets being the ER and mitochondria. Our studies demonstrate that one of the short-term effects of endogenous NO production is enhanced sensitivity for the CD95 apoptosis pathway. Prolonged exposure to NO induced ER and mitochondrial stress causing activation of caspase-4 and -9 leading to apoptosis. Furthermore, cytokines, secreted by lymphokine-activated killer cells, can induce endogenous NO synthesis and apoptosis in colon cancer cells (53). This non-contact-dependent cell toxicity mechanism supports a role for NO in T cell-mediated tumor defense.

Our results highlight a paradoxical role of NO during tumorigenesis. On one hand, NO induces apoptosis and sensitizes cells to apoptosis mediated by CD95, and on the other hand, NO promotes survival of tumor cells. On the basis of our results, we propose that the apoptosis-enhancing effect of NO initially reduces tumor formation but finally results in the elimination of cells with a fully functional apoptotic response and the retention of a subpopulation of cells with an aberrant or attenuated response to death-inducing signals. Chronic exposure to NO thus facilitates the clonal evolution of a population of cells that can circumvent normal death-inducing signals, including those derived from CTLs during immune surveillance. One of the hallmarks of cancer cells is apoptosis resistance. We postulate selection for apoptosis-resistant tumor cells may be the product of repeated genomic and cellular insults due to continuous NO exposure. Analysis of the cell clones we have obtained during these experiments will allow us to further characterize the molecular mechanisms involved in developing NO and apoptosis resistance in tumor cells.

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Disclosures
The authors have no financial conflict of interest.

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