Endogenously produced nitric oxide mitigates sensitivity of melanoma cells to cisplatin
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Melanoma patients experience inferior survival after biochemotherapy when their tumors contain numerous cells expressing the inducible isoform of NO synthase (iNOS) and elevated levels of nitrotyrosine, a product derived from NO. Although several lines of evidence suggest that NO promotes tumor growth and increases resistance to chemotherapy, it is unclear how it shapes these outcomes. Here we demonstrate that modulation of NO-mediated S-nitrosation of cellular proteins is strongly associated with the pattern of response to the anticancer agent cisplatin in human melanoma cells in vitro. Cells were shown to express iNOS constitutively, and to generate sustained nanomolar levels of NO intracellularly. Inhibition of NO synthesis or scavenging of NO enhanced cisplatin-induced apoptotic cell death. Additionally, pharmacologic agents disrupting S-nitrosation markedly increased cisplatin toxicity, whereas treatments favoring stabilization of S-nitrosothiols (SNOs) decreased its cytotoxic potency. Activity of the proapoptotic enzyme caspase-3 was higher in cells treated with a combination of cisplatin and chemicals that decreased NO/ SNOs, whereas lower activity resulted from cisplatin combined with stabilization of SNOs. Constitutive protein S-nitrosation in cells was detected by analysis with biotin switch and reduction/chemiluminescence techniques. Moreover, intracellular NO concentration increased significantly in cells that survived cisplatin treatment, resulting in augmented S-nitrosation of caspase-3 and prolyl-hydroxylase-2, the enzyme responsible for targeting the protein S-nitrosation is associated with the phenotype of cells to apoptosis (13), which is dependent on S-nitrosation (14, 15), the covalent binding of NO to protein cysteine residues that leads to functional alterations. This posttranslational modification accounts for guanylyl cyclase-independent signaling properties of low levels of NO, and has been involved in the regulation of an increasing number of biological processes (13, 16–18). Imbalanced S-nitrosation is linked to the etiology of several diseases (17), and it has been suggested that cancer cells may exploit this biomolecular modification to escape cell death (18).

A strong correlation has been shown between the prevalence of tumor cells expressing inducible NOS (iNOS; i.e., NOS2) and shortened survival of patients with advanced melanoma (6, 9, 12, 19, 20). Constitutive expression of iNOS has been detected in most metastatic melanomas and melanoma cell lines (9, 19, 20), and it has been suggested that NO contributes to tumor survival (12, 21). In addition, inhibition of NO production increases sensitivity to cisplatin in vitro (22) and acts synergistically with cisplatin to reduce tumor development in experimental melanoma (23). However, molecular mechanisms through which endogenous NO protects tumors have not been defined. Here we show that NO produced endogenously by melanoma cells is an important regulator of their biology and decreases their sensitivity to cisplatin. Moreover, our results indicate that increased protein S-nitrosation is associated with the phenotype of cells able to escape drug-induced apoptosis.

Results

Based on clinical and experimental evidence correlating NO with poor response to cisplatin (15, 21–23), we initially confirmed that endogenously generated NO promotes resistance to cisplatin-induced apoptosis in three human melanoma cell lines. First, we demonstrated constitutive expression of iNOS by immunoblot (Fig. 1D). Expression of other NOS isoforms was inconsistent or not detected. Although NO was not secreted at detectable amounts into culture medium by melanoma cells, analysis of whole-cell extracts by reduction/chemiluminescence using an NO analyzer showed that NO was present at low nanomolar levels (Fig. 1B and C). Complementary studies with the fluorescent dye 4,5-diaminofluorescein diacetate (DAF-2DA) confirmed the presence of NO and its derivatives in intact, live cells (Fig. S14), as well as decreased intracellular NO upon incubation with the iNOS inhibitor 1400W (Fig. S1B).

To assess impacts of endogenous NO on the physiology of melanoma cells, growth was evaluated in cells cultured in presence of the broad-spectrum NOS inhibitor N-nomonomethyl-arginine (NMA), which resulted in reduced growth in all cases (Fig. 1D). Sensitivity to cisplatin was also affected by endogenous NO, as shown by enhanced killing in cells treated with NMA (Fig. 1E). Similar results were seen in A375 cells treated with 2- (4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (APTDI).


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(PTIO; which scavenges NO-derived species) or with the iNOS inhibitor 1400W (Fig. S2) and subsequently challenged with cisplatin (Fig. 1F). Cells treated with an inactive form of PTIO before challenge with cisplatin displayed the same extent of cell death as those treated with cisplatin alone, confirming that NO was involved (Fig. 1F). Further, annexin V staining showed that scavenging of NO led to increased apoptosis and significantly enhanced the toxicity of cisplatin (Fig. 1G).

We hypothesized that protein modification by S-nitrosation was a potential mechanism through which NO exerted the aforementioned effects. Results supporting that hypothesis showed that pretreatment of cells with dimitrochlorobenzene (DNCB), which favors the stabilization of S-nitrosothiols (SNOs) by inhibiting the activity of thioredoxin reductase (24–26), increased survival of A375 cells challenged with cisplatin (Fig. 2A). Conversely, transient treatment of cells with a nontoxic concentration of the reducing agent DTT to disrupt SNOs markedly increased cisplatin toxicity in a dose-dependent manner (Fig. 2B), corroborating the findings obtained with DNCB and suggesting S-nitrosation as a regulator of chemosensitivity. Pretreatment with DTT also increased sensitivity to cisplatin, rendering cells responsive to concentrations that did not cause cell death in the absence of the reducing agent (Fig. 2C).

We subsequently investigated cell signaling targets potentially affected by NO during these responses. Immunoblot analysis showed that activation of Akt by phosphorylation, a survival signal, was not affected by an NMA-mediated decrease in NO concentration during the response to cisplatin in A375 or SK Mel 28 cells (Fig. 3A). Similarly, expression of p53 and its downstream regulator MDM2, both of which are involved in responses to DNA damage, was not significantly affected by decreased NO production. Phosphorylation activation of NFkB p65 was changed only in SK Mel 28 cells when NMA was combined with cisplatin. On the contrary, ERK1/2 was clearly activated in both cell lines, as indicated by increased phosphorylation when cells were exposed to a combination of NMA and cisplatin (Fig. 3A), a treatment that resulted in enhanced cell death (Fig. 1E).

As ERK1/2 activation is essential for induction of apoptosis by cisplatin (4, 27, 28), expression of a number of pro- and antiapoptotic proteins was determined using the same experimental conditions (Fig. 3A). As shown in Fig. 3B, NOS inhibition with NMA during challenge with cisplatin in A375 cells reduced the levels of the key antiapoptotic protein Bcl-2, whereas the opposite effect was observed for proapoptotic proteins Bax and, more strikingly, PUMA. These results show that NO was a critical component in the regulation of cisplatin-induced cell death in this model.

Because the apoptotic effector enzyme caspase-3 is a known target for inhibition by S-nitrosation (29), we investigated whether NO effects on cisplatin-induced cell death were attributable to changes in its activity resulting from modulation of S-nitrosation. Indeed, A375 cells grown in presence of NMA to reduce NO production and challenged with cisplatin displayed 20% higher caspase-3 activity than cells treated with cisplatin alone (Fig. 4A). Similarly, disruption of SNOs by a nontoxic dose of DTT enhanced cisplatin-induced caspase-3 activity early after addition of cisplatin (Fig. 4B). On the contrary, stabilization of S-nitrosation with DNCB inhibited 50% of caspase-3 activity in response to cisplatin (Fig. 4C). These data suggest that S-nitrosation is a possible mechanism underlying the effects of NO, partially mitigating cisplatin toxicity by inhibiting caspase-3 activity and interfering with apoptosis.

In view of results indicating that NO fosters chemoresistance, we investigated the hypothesis that increased production of NO induced by cisplatin contributed to enhanced survival. Analysis of A375 cells surviving a 24-h treatment with cisplatin revealed a significant increase in NO content (Fig. 5A and B), supporting this interpretation; concurrent treatment with the NOS inhibitors NMA and L-N6-(1-Iminoethyl)lysine hydrochloride (L-NIL) completely eliminated detectable levels of NO in cell homogenates.

To verify whether the increment in NO production induced by cisplatin had the potential to modulate cell signaling via a corresponding increase in protein S-nitrosation, we next determined levels of S-nitrosation in proteins known to be targets of this modification by using the biotin switch technique. Analysis of proteins isolated from cell extracts showed a twofold increase in
S-nitrosation of caspase-3 in response to cisplatin in A375 and SK Mel28 cells (Fig. 5C). In addition, prolyl-hydroxylase 2 (PHD2), the enzyme that targets the prosurvival transcription factor hypoxia-inducible factor-1α (HIF-1α) for degradation, displayed a fivefold increase in S-nitrosation upon cisplatin challenge (Fig. 5D), reflected in a corresponding accumulation of HIF-1α (Fig. 5E). Therefore, increased S-nitrosation of enzymes involved in apoptosis (caspase-3) and survival (PHD2), both known targets for inhibition by this modification (29, 30), confirms an association between S-nitrosation and increased drug-resistance.

**Discussion**

Low levels of endogenous or exogenous NO enhance progression of cancer in vitro and in vivo (7, 13, 18, 21, 23, 31). Strong clinical and experimental evidence shows a correlation between production of NO by tumor cells and reduced survival of patients with advanced melanoma (11, 19, 32), as well as poor response to chemotherapy and radiation therapy (17, 22, 23, 33, 34). The matrix of regulatory mechanisms underlying these interactions is undoubtedly multifactorial and complex. Although NO can promote angiogenesis (11) and tumor-protective mutations (9, 13), the focus of the present study was to investigate the role of low levels of endogenously produced NO in signaling pathways affected by treatment with cisplatin. Our findings confirm earlier reports of constitutive synthesis of NO by human metastatic melanoma cells and extend them by showing that NO production results in muted responses to cisplatin in vitro, with S-nitrosation representing a potentially important biochemical way through which it exerts these effects.

The role of NO in tumorigenesis and tumor progression has been an object of intense investigation, stimulated by the expression of NOS in many types of cancer cells, its correlation with reduced patient survival, and its potential as a therapeutic target (11, 13, 19). NOS expression by tumor cells has been an object of intense investigation, stimulated by the expression of NOS in many types of cancer cells, its correlation with reduced patient survival, and its potential as a therapeutic target (11, 13, 19). NOS expression by tumor cells has been...
levels of cytokine- or viral vector-driven expression and activity of NOS have been shown to lead to tumor cell killing, usually requiring the participation of proinflammatory factors, such as TNF-α, γ-IFN, and IL-1 (7, 21). At high concentrations, NO produced by stromal, immune, or cancer cells themselves causes nitrosative stress, which leads to tumor death by DNA and protein damage (9). In contrast, noninduced, i.e., constitutive, expression of iNOS results in production of low, nontoxic levels of NO (15), which favors growth and progression of tumor cells, as our present results and other reports (13, 16, 23) demonstrate. We could not detect NO production by melanoma cells using the Saville–Griess assay, but were able to detect and quantify it by using an NO analyzer and DAF-2DA, both of which are sensitive to nanomolar concentrations of NO and its metabolites. Under these circumstances, the signaling qualities of NO are of increased importance, potentially modulating protein activity within signaling pathways by S-nitrosation, which may, for example, contribute to the relative resistance of melanoma cells to cisplatin-induced apoptosis, as our data indicate.

Lack of information specifically addressing the molecular basis for tumor-protective properties of NO prompted us to investigate the involvement of its signaling properties in the response of melanoma cells to cisplatin. A key feature of chemoresistance is the ability of cancer cells to resist drug-induced apoptosis (1, 4, 7, 18). Of note, S-nitrosation is known to regulate apoptosis in cancer cells, whereas these effects of NO have not been observed in normal melanocytes (12, 13, 16). This reinforces the proposition that tumor cells may exploit S-nitrosation–based mechanisms to resist cell killing (18). Our results show that suppression of NO production during treatment with cisplatin led to increased activation of ERK1/2 and enhanced cell death compared with cisplatin alone. Even though ERK1/2 activation has been linked to survival signaling, a number of reports have shown that this kinase complex is critical for induction of apoptosis by platinum agents (4, 27, 28), which induce anomalous, sustained hyperactivation of ERK1/2 (28, 36). In fact, we observed that conditions leading to augmented ERK1/2 activation and increased apoptosis also decreased expression of the antiapoptotic protein Bcl-2 and up-regulated expression of the proapoptotic factors PUMA and Bax, which function in association in the initiation of apoptosis (37). These data support the interpretation that cell fate after chemotherapy depends on alteration of the balance between anti- and prosurvival components (4, 12), and show that NO plays a key role in the process. Collectively, our findings clearly show an association between NO-induced S-nitrosation and altered cytotoxicity of cisplatin in melanoma cells. In themselves, however, they do not provide definitive evidence that S-nitrosation is responsible for effects on signaling pathways such as that shown in Fig. 3; this will require further investigation.

In addition to the altered levels of proteins involved in the response to cisplatin, we found that, under the same condition, caspase-3 and PHD2 displayed increased S-nitrosation, a protein modification known to inhibit their functions (29, 30, 34). Caspases are the ultimate effectors of apoptosis, and their activity is attenuated in cells resistant to cisplatin (4). NO interferes with caspase activity by S-nitrosation of the active site (29), and also by impairing the cleavage of proenzymes, which is required for their activation (16, 24). On the contrary, HIF-1α, whose degradation is governed by PHD2, favors several aspects of tumorigenesis, including resistance to chemotherapy and radiation therapy (38, 39). NO promotes stabilization of HIF-1α directly, via S-nitrosation of its oxygen-dependent domain (33), or indirectly, by inhibiting PHD2 (30, 34). Our data show that cisplatin induces accumulation of HIF-1α, which could be partially attributable to the observed increased S-nitrosation inhibition of PHD2. Our findings of inducible increases in S-nitrosation of caspase-3 and PHD2 are previously unrecognized features of the complex mechanisms of drug resistance. Additional research is needed to confirm the significance of S-nitrosation of these proteins in the context of numerous other potential regulatory mechanisms (1–5, 11, 40).
We showed that, in response to cisplatin, melanoma cells increased NO levels and up-regulated S-nitrosation of specific proteins in a fashion that promoted survival signals (HIF-1α accumulation via S-nitrosation inhibition of PHD2) and inhibited apoptosis (through S-nitrosation inhibition of caspase-3 activity). S-nitrosation and denitrosation of cellular proteins are regulated through an enzymatic complex, in which thioredoxins, thioredoxin reductase, thioredoxin-interacting protein, and S-nitrosoglutathione reductase orchestrate the cellular pool of SNOs and, therefore, protein function (24, 41). In addition, the fact that S-nitrosation of a given protein depends on its subcellular localization, rather than solely on the amount of available NO, further emphasizes that S-nitrosation is a finely controlled mechanism (17, 29, 41), an imbalance of which is linked to genesis of several pathologic states (13, 16–18, 41). We conjecture that, during the response to cisplatin, a process is activated in which NO concentration increases and SNO levels are modulated by the aforementioned enzymes (together with other mechanisms yet to be identified), enabling cancer cells to mitigate apoptosis.

To characterize the regulation of cisplatin-induced apoptosis by S-nitrosation, we used DTT and DNCB to promote disruption and stabilization of intracellular SNOs, respectively. By using a similar approach, other investigators employed DTT to show the involvement of S-nitrosation of proteins such as caspases (43), Bcl-2 (18), and PHD2 (30). DNCB, which irreversibly inhibits thioredoxin reductase (25, 26), has been shown to increase the amount of S-nitrosylated caspase-3 (24) and PHD2 (30). Of note, because these compounds do not affect exclusively SNOs (i.e., DTT) or thioredoxin reductase (i.e., DNCB), other concurrent effects of these chemicals cannot be ruled out. However, interpretation of our results as evidence of S-nitrosation–mediated phenomena is supported by the responses to NOS inhibitors (NMA, L-NIL, 1400W) and the NO scavenger PTIO as well. Although it has been shown that PTIO may promote oxidation of NO and even formation of the nitrosating agent N2O3 under certain conditions (44), those used in our investigation do not support such chemistry.

Our finding that decreased NO concentrations (and possibly disruption of SNOs) substantially increase sensitivity of cells to cisplatin is particularly noteworthy. Agents of the platinum family are highly toxic and induce serious side effects in renal, neural, myeloid, and auditory tissues (5), considerably limiting their usefulness and emphasizing the need for means to optimize their efficacy. Several investigators have suggested therapeutic protocols combining NO-decreasing strategies with cisplatin or other anticancer drugs (21, 23, 30, 31, 33, 34). Protocols already in clinical use, which include N-acetylcysteine, rosiglitazone, iNOS antisenes oligomers, and physical activity, reportedly decreased S-nitrosation in other pathologic conditions (17). Therefore, further exploration of combination of cisplatin with NO-decreasing (denitrosating?) agents may be a promising strategy to potentiate its desirable effects while diminishing side effects by enabling the use of lower doses.

Materials and Methods

Chemicals and Antibodies. Cisplatin (cis-diaminedichloroplatinum(II)), DNCB, and cisplatin were purchased from Sigma-Aldrich. NMA and PTIO were from EMD-Calbiochem. Antibodies were as follows: iNOS, caspase-3, PUMA, and Bcl-2 from Santa Cruz; p53 and β-actin from EMD-Calbiochem; HIF-1α from BD Transduction Laboratories, MDM2 from Millipore, and PHD2 from AbCam. All other antibodies were from Cell Signaling Technologies.

Cell Culture. Human melanoma cell lines A375, SK Mel 28, and SK Mel 100 (provided by Elizabeth A. Grimm, MD Anderson Cancer Center, Houston, TX) were grown at 37 °C and 5% (vol/vol) CO2 in DMEM (A375) or RPMI (SK Mel cells) supplemented with 10% (vol/vol) FBS (Atlanta Biologicals), 0.2 mM l-glutamine, 10 U/mL penicillin, 10 μg/mL streptomycin, and 1 mM sodium pyruvate (Lonza).

Cell Proliferation and Cytotoxicity. WST-1 reagent (Roche) was used to analyze cell growth and viability. Ten microliters of reagent was added per 100 μL of culture medium, and the absorbance was read at 450 nm after incubation for 30 min at 37 °C.

Quantitative Analysis of NO. Cells were grown in medium containing 12.5 μM cisplatin for 24 h. For measurement of NO content in homogenates, cells were washed in PBS solution at 4 °C and lysed in PBS solution containing 1% Nonidet P-40, 2 mM N-ethylmaleimide, 0.2 mM diethylenetriaminepentaacetic dihydride, and protease inhibitors. After three freeze-thaw cycles (–80°C), lysates were passed through a 29-gauge needle to reduce viscosity and spun at 2,000 × g for 10 min at 4 °C. Protein concentration was measured in the soluble phase, and aliquots containing 2 mg of protein were brought up to 100 μL with lysis buffer and kept on ice for analysis.
S-Nitrosation was detected by the biotin switch technique as described elsewhere (43), with modifications. Unless otherwise stated, all reagents were from Sigma. After treatments, cells were rinsed with PBS solution containing 1 mM EDTA and 0.1 mM nuccepeoine, followed by lysis in 25 mM HEPES, pH 7.4, 50 mM NaCl, 0.1 mM EDTA, 1% Nonidet P-40, and protease inhibitors. From each sample, 0.5 mg of total protein was used for the assay. The volume of samples was adjusted to 650 μL with HEN buffer (100 mM Hepes, pH 8, 1 mM EDTA, 0.1 mM nuccepeoine) and SDS 2.5% (wt/vol; final concentration). Free thiols were blocked with methylmethanethiosulfonate (0.15% final concentration) and incubation for 30 min at 25 °C and vigorous vortexing. Excess methyl methanethiosulfonate was removed by acetone precipitation and gentle rinse of protein pellets with chilled 70% (vol/vol) acetone, followed by resuspension in HEN buffer containing 1% SDS. Biotinylation of SNOs was performed with a final concentration of 50 μM [N-(6-Biotinamido)hexyl]-3′-(2-pyrildithio)-propionamide) [EZ-Link Biotin-HPD-P, in DMSO; Thermo Scientific] and 20 mM sodium ascorbate (final concentration), incubated under gentle rotation for 1 h at room temperature. For each sample, a parallel control for specificity of S-nitrosation detection was performed in which ascorbate was omitted from the reaction. Until this step, all procedures were performed in the dark. Unbound biotin was removed by acetone precipitation as described above. Pellets were resuspended in HEN/10 buffer (1/10 HEN buffer plus 9/10 water and 1% SDS) and three volumes of neutralization buffer (25 mM Hepes, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100). Full-drown of biotinylated (i.e., nitrated) proteins was carried out by overnight incubation with NeutrAvidin Agarose beads (Thermo Scientific) at 4 °C with gentle rotation. Beads were washed by centrifugation with neutralization buffer containing 600 mM NaCl. Biotinylated proteins were eluted under reducing conditions, and detection of specific proteins was done by immunoblot.

Statistical Analysis. All results are from experiments performed at least three times with similar results. Figures show results representative of each experiment. Paired data were analyzed with the unpaired t test. Multiple treatments were analyzed by one-way ANOVA and complemented by the Student–Newman–Keuls multiple comparisons test.

NO Detection and Analyses of Apoptosis. Details concerning NO detection with DAF-2DA and analyses of apoptosis are provided in SI Materials and Methods.

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