NO Signaling Confers Cytoprotection through the Survivin Network in Ovarian Carcinomas

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Abstract
Despite considerable success in the treatment of epithelial ovarian cancer (EOC), therapy resistance counteracts improvement of long-term survival. The dual role of survivin as an apoptosis inhibitor and mitotic regulator has been associated with disease outcome. However, the molecular mechanisms involved in the deregulated expression in EOC of survivin need further investigation. Here, we show that high amounts of the nitric oxide (NO) donors, S-nitroso-N-acetylpenicillamine (SNAP) and sodium nitroprusside (SNP) or strong overexpression of the inducible nitric oxide synthase (iNOS) suppressed survivin levels via the p38MAPK pathway and triggered apoptosis in ovarian cancer cell lines (OCC). Importantly, low NO concentrations conferred resistance against carboplatin/paclitaxel-induced apoptosis. Cytoprotection was mediated by survivin because we observed its upregulation subsequently to low SNAP/SNP doses or ectopic expression of low amounts of iNOS. Also, RNAi-mediated depletion of survivin blocked the antiapoptotic effects of NO signaling. Induction of survivin involves activation of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway, which was antagonized by the PI3K-inhibitor, LY294002. Interestingly, application of the iNOS-inhibitor 1400W together with RNAi-mediated survivin down-regulation cooperatively enhanced drug-induced apoptosis in OCCs. The iNOS/survivin interdependencies seem to be also of clinical relevance because immunohistochemistry revealed that low iNOS levels correlate with survivin expression (P < 0.01) in carboplatin/paclitaxel-treated EOC patients with minimal postoperative residual tumor (n = 54). Also, iNOS and survivin expression were associated with increased risk for disease progression. Our study uncovers a novel molecular mechanism of how NO signaling may contribute to therapy resistance in EOC by modulating survivin expression. Pharmacogenetic iNOS/survivin-targeting strategies may hence be pursued to complement current treatment modalities in EOC. [Cancer Res 2008;68(13):5159–66]

Introduction
Although ovarian cancer is only the fifth most common cancer among women, it is the leading cause of gynecologic cancer deaths (1). Due to the insidious, nonspecific generalized symptoms associated with the disease, most patients present with stage III or IV disease. After primary surgery, the current standard treatment for patients with advanced epithelial ovarian cancer (EOC) involves the systemic administration of a paclitaxel/platinum-containing chemotherapy regimen (2). Despite the enthusiasm for optimal cytoreductive surgery and combination chemotherapy with intent to cure, >50% of patients die within 5 years of their initial diagnosis (ref. 2, and references within). In contrast to high response rates to first-line chemotherapy, EOC is further characterized by the rapid development of resistance to treatment leading to recurrences in most patients.

Among the multifaceted molecular events responsible for resistance in EOC, overexpression of inhibitor of apoptosis proteins (IAP; refs. 3, 4) and nitric oxide (NO) signaling (5, 6) seem to influence tumor cell apoptosis and disease outcome.

The diffusible molecular messenger NO is synthesized endogenously from -arginine by three isoforms of the enzyme NO-synthase. NO signaling participates in many physiologic and pathophysiologic processes, including cancer (details refs. 5, 6). The biological effects of NO are ultimately determined by redox chemistry and derived reactive nitrogen oxide species. NO can affect various cellular functions through posttranslational modifications of proteins and the guanylate cyclase-induced activation of signaling networks (see refs. 5, 6). Low (<50 nmol/L) and intermediate NO levels (100–300 nmol/L) are produced by neuronal nitric oxide synthase (NOS) and endothelial NOS, whereas high levels (>300 nmol/L) are attributed to inducible NOS (iNOS; ref. 6). iNOS is the isoform most consistently associated with EOC. Several studies implicate that high NO concentrations (in the micromole range) generated by chemical NO donors or enforced overexpression of iNOS may be used to kill cancer cells (see refs. 5, 6). Paradoxically, iNOS is overexpressed in many tumors, including EOC, and overexpression of iNOS often correlates with bad prognosis (see refs. 5–7). Hence, NO may also promote tumor progression via induction of tumor-cell proliferation and antiapoptotic programs, as well as through the expression of angiogenic factors (see refs. 5, 6). Thus, although the expression of iNOS has been studied in gynecologic tumors and cell lines, its functional and prognostic significance are still not fully understood (see refs. 5, 7, 8).
Among the IAPs overexpressed in cancer, its proposed dual role as an apoptosis inhibitor and a mitotic effector positioned survivin in the front line of cancer research (reviewed in refs. 3, 4, 9). Survivin is largely undetectable in differentiated tissues but highly expressed in most human neoplasms, including EOC (10). Survivin is a dynamic protein capable to shuttle between the nucleus and the cytoplasm (11). Its expression has been reported to correlate with reduced tumor cell apoptosis, increased resistance to chemotherapy, and abbreviated patient survival (12, 13). Several signal transduction pathways (e.g., Her-2/neu, ER, epidermal growth factor receptor, etc.) seem to contribute to deregulating the cell-cycle–dependent transcription of survivin, and/or to enhancing the half-life and function of survivin by posttranslational modifications (see refs. 3, 9, 10, 14). However, whether and how NO signaling may affect survivin expression and its functional consequences in ovarian carcinomas remain yet to be determined.

To better tailor current treatments and to develop translational therapeutic strategies for EOC patients, an improved molecular understanding of therapy resistance and recurrence together with the identification of prognostic and predictive factors is of utmost importance. Because histologic subtypes of ovarian tumors are likely to be associated with distinct morphologic and molecular profiles (14, 15), and residual disease after primary surgery is one of the strongest predictors for survival (reviewed in ref. 16), we here focused exclusively on serous EOC patients with macroscopic complete surgical tumor resection.

Corroborating experimental approaches using ovarian cancer cell lines revealed that NO signaling is capable of stimulating survivin expression, thereby conferring enhanced resistance to carboplatin/paclitaxel. In addition, we show for the first time that NO can enhance or repress survivin expression in a concentration-dependent manner by stimulating either the phosphatidylinositol-3-kinase (PI3K)/Akt or p38MAPK-kinase (p38MAPK) pathway, which ultimately modulates the cytoprotective cellular response. Our results further uncover the prognostic and functional relevance of the iNOS and survivin interdependencies in advanced-stage serous ovarian carcinomas after radical surgery and postoperative chemotherapy.

Materials and Methods

Patients and treatment. The study included 54 primary ovarian carcinoma patients treated between 1999 and 2004 at the Departments of Gynecologic Oncology and Gynecology in Wiesbaden and the University of Frankfurt. Studies of human tissue biopsies were approved by the Local Research Ethics Committees, and samples were processed anonymously. Inclusion criteria were serous EOC and macroscopic complete debulking (see refs. 3, 9, 10, 14). However, whether and how NO signaling may affect survivin expression and its functional consequences in ovarian carcinomas remain yet to be determined.

Plasmids. The eukaryotic expression vector pc4-iNOS was constructed by reverse transcription-PCR amplification of the human iNOS coding sequence using specific oligonucleotides (iNOS_cds_5P, 5'-GGGACATCTC-GAGATGCCGGCTCCATTG-3'; iNOS_cds_3P, 5'-CCGCGGCCTGTACATC-3') and cloning into the BglII- and NotI-digested expression vector pcDNA4/TO (Invitrogen). Ectopic survivin expression was achieved by using the retroviral expression vector M387-survivin (15).

RNAi. RNAi-mediated ablation of survivin was performed using double-stranded siRNAs (Eurogentec) as described in detail (11).

Immunoblotting, immunofluorescence, and antibodies. Immunoblotting and immunofluorescence were carried out according to Kramer and colleagues (19). To control equal loading of cell lysates, blots were reprobed with α-actin Abs as described (19). Abs were as follows: α-(anti)-Akt and α-phosphoheresine 473 Akt (NEB), α-p38MAPK and α-phospho-threonine 180/182-p38MAPK (Cell Signaling Technology, Inc.), α-survivin (NB-500-201; Novus Biologicals), α-iNOS (rabbit polyclonal; provided by J. Pfeilschifter, University of Frankfurt, Frankfurt, Germany), and appropriate Cy3/FITC-conjugated secondary Abs (Santa Cruz Biotechnology).

Microscopy. Observation of living or fixed cells and image analysis were performed as described in detail (11, 20).

Reagents and treatment. The PI3K/Akt inhibitor, LY294002 (15 μmol/L), and the p38MAPK inhibitor, SB203580 (15 μmol/L), were from Calbiochem. The NO donors 5-nitros- N-acetyl-penicillamine (SNAP) and sodium nitroprusside (SNP) were purchased from Sigma Aldrich. Cells were treated with cisplatinum (40 μmol/L) or Taxol (50 μmol/L; Sigma Aldrich) as described (18). Inhibition of iNOS enzymatic activity was performed by culturing cells in the presence of 50 μmol/L of the specific iNOS inhibitor N-3-(aminomethyl)benzylacetamide (1400W; AXXORA GmbH).

Nitrite measurement. NO production was analyzed by measuring nitrite by the Griess reaction in cultured medium according to (21).

Measurement of apoptosis, cell cycle, and viability. 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, propidium iodide–fluorescence-activated cell sorting analyses and assessment of apoptosis by quantitation of caspase-3 activity in cell extracts were performed as described in detail (13, 22).

Results

Effects of NO on cytotoxicity and chemotherapy-induced apoptosis in ovarian carcinoma cell lines. As the majority of chemotherapeutic drugs cause apoptosis, and NO signaling has been shown to have a proapoptotic as well as an antiapoptotic mediator, we set out to study the biological effects of NO on the ovarian carcinoma cell lines (OCC), OVCA-1, and SKOV-3. First, we used synthetic NO donors (SNAP, 1 μmol/L; SNP, 2 μmol/L) to induce cell death, whereas high NO concentrations scored semiquantitatively based on staining intensity and distribution using the immunoreactive score (IBS; ref. 13). All assessments were performed blinded with respect to clinical patient data.

Statistical analysis. Time-to-event outcomes (progression-free survival; PFS) were analyzed with the Kaplan-Meier product-limit method and log-rank test as described (13). Association between different factors was measured with the Spearman’s rank correlation coefficient r. For all cell culture experiments where P values are expressed, a paired Student’s t test was performed, representing data from three independent experiments done in triplicate. P values of <0.05 were considered as significant.

Cells, transfection and transduction. The ovarian carcinoma cell lines OVCA-1 and SKOV-3 were maintained under conditions recommended by the American Type Culture Collection. Human umbilical vascular endothelial cells (HUVEC) were isolated from term umbilical cord veins and cultured according to (17). Cells were transfected or transduced as described (13). Cells stably expressing iNOS were established by G418 selection according to (18).

Materials and Methods

Patients and treatment. The study included 54 primary ovarian carcinoma patients treated between 1999 and 2004 at the Departments of Gynecologic Oncology and Gynecology in Wiesbaden and the University of Frankfurt. Studies of human tissue biopsies were approved by the Local Research Ethics Committees, and samples were processed anonymously. Inclusion criteria were serous EOC and macroscopic complete debulking during primary surgery. The majority of patients had advanced disease stages [Fédération Internationale des Gynécologues et Obstétristes (FIGO) III–IV] and had received primary surgery followed by platinum- and taxane-based chemotherapy (Supplementary Table S1) according to du Bois and colleagues (2). The median follow-up time was 96 wk (range, 0–297 wk).

Tissue samples and immunohistochemistry. Tissue samples were fixed and paraffin embedded as described (13). Sections were used for primary diagnosis, second reviewing and for tissue microarray (TMA) construction according to Engels and colleagues (13). TMA sections were processed and stained as described (13). For antigen retrieval, sections were pretreated in a pressure cooker with EDTA buffer (10 mmol/L; pH 8.0) for survivin or treated in a microwave oven with EDTA buffer (10 mmol/L; pH 9.0) for iNOS. For visualization of survivin and iNOS, the EnVision detection system (Dako GmbH) was applied as described (13). Sections were counterstained with hematoxylin. For negative controls, the primary antibodies (Ab) were omitted. Expression levels for iNOS and survivin were
SNAP, 0.5 mmol/L; SNP, 1 mmol/L) resulted in the inhibition of cell growth (Fig. 1A) and increased G2-M arrest (data not shown). Importantly, low NO levels (SNAP, 0.1 mmol/L; SNP, 0.2 mmol/L) reduced the cytotoxic effects of treatment with CDDP or Taxol, whereas increased programmed cell death (PCD) was observed upon their combination with high doses of NO donors (SNAP, 0.5 mmol/L; SNP, 1 mmol/L; Fig. 1B).

NO-induced modulation of cell survival is mediated by survivin. Because we and others (see refs. 4, 9) have shown that expression of survivin-protected cancer cells from drug-induced cytotoxicity, we examined whether the observed cytoprotective effect of low NO concentrations was mediated by the up-regulation of survivin. Immunoblot analysis revealed that treatment with low amounts of NO donors indeed increased survivin expression in unsynchronized OCCs (Fig. 1C). In contrast, survivin was significantly reduced when the cells were exposed to high concentrations of NO donors, which correlated with increased PCD and reduced proliferation (Fig. 1A and B). To further confirm that survivin counteracts NO-induced apoptosis in OCCs, we ectopically expressed survivin by retroviral transduction and challenged the cells with cytotoxic amounts of NO donors. In contrast to cells transduced with internal ribosomal entry sequence–green fluorescent protein (IRES-GFP), apoptosis was ameliorated by increased survivin levels (Fig. 1D). Enhanced survivin expression in survivin-IRES-GFP–transduced cells was verified by immunoblot analysis (Fig. 1D).

p38MAPK is involved in NO-mediated apoptosis and survivin repression. As reviewed by (5, 6), the p38MAPK pathway seems to be involved in several aspects of NO signaling. We also observed an up-regulation and activation of p38MAPK upon treatment with cytotoxic doses of NO donors (SNAP, 0.5 mmol/L; SNP, 1 mmol/L; Fig. 2A). Next, we used the p38MAPK-specific inhibitor, SB203580, and examined its effect on NO-induced PCD and survivin expression. OCCs were pretreated with SB203580

**Figure 1.** Effects of NO donors on cytotoxicity and chemotherapy-induced apoptosis in OCCs is mediated by survivin. Apoptosis was assayed by measuring caspase-3 activity, which was set to 1 in untreated cells. Columns, mean from three independent experiments; bars, SD. Indicated P values were calculated using the two-sided Student’s t test. A, SCOV-3 cells were incubated with the indicated concentrations of SNAP or SNP, and proliferation was analyzed by the MTT assay. Whereas low amounts of NO donors (SNAP, 0.1 mmol/L; SNP, 0.2 mmol/L) stimulated cell proliferation, high NO concentrations (SNAP, 0.5 mmol/L; SNP, 1 mmol/L) resulted in inhibition of cell growth. B, SCOV-3 cells were treated with the indicated concentrations of SNAP or SNP for 24 h. Subsequently, Taxol or CDDP were added, and apoptosis was assayed 24 h later. Low NO donor concentrations reduced drug-induced apoptosis, whereas high NO levels resulted in increased PCD. C, NO-induced increase of cell survival is mediated by survivin. Survivin protein levels were up-regulated in SCOV-3 cells treated with low concentrations of NO donors, whereas survivin expression was reduced upon exposure to high amounts of SNAP/SNP. Survivin protein was detected by immunoblotting with α-survivin Abs. Reprobing with α-actin Abs confirmed equal loading of cell lysates. D, ectopic expression of survivin by retroviral transduction reduced the cytotoxic effects of NO donors. Twenty-four hours after infection, OVCAR-3 cells expressing survivin-IRES-GFP or IRES-GFP were incubated with the indicated NO donors, and apoptosis was assessed 24 h posttreatment by measuring caspase-3 activity. Immunoblotting with α-survivin Abs confirmed enhanced survivin expression in survivin-IRES-GFP transduced OVCAR-3 cells. Actin served as loading control.
(15 μmol/L, 3 hours) and incubated with NO donors (SNAP, 0.5 mmol/L; SNP, 1 mmol/L) for 24 hours. Subsequently, apoptosis and survivin expression were assayed by caspase-3 activity and immunoblot, respectively. As shown in Fig. 2B, NO-induced PCD was significantly reduced by SB203580. Likewise, NO-mediated reduction of survivin levels was counteracted by pharmacologic inhibition of the p38MAPK pathway (data not shown; Fig. 2C).

**iNOS can modulate chemotherapy-induced PCD via survivin expression in OCCs.** Next, we tested whether the biological effects observed upon exogenous NO administration can be reproduced by stimulating endogenous NO levels. Therefore, we ectopically expressed iNOS in OCCs and examined its consequences on survivin expression and apoptosis. Immunoblot analysis verified that transfection of increasing amounts of pc4-iNOS correlates with enhanced iNOS protein levels (Fig. 3A). Similar to the results obtained by chemical NO induction, we observed an increase of survivin levels upon expression of low amounts of iNOS, whereas strong iNOS overexpression resulted in reduced survivin levels. Subsequently, the transfected cells were challenged with cytotoxic drugs, and apoptosis was quantitated by assaying caspase-3 activity. Compared with cells transfected with the empty vector, the cytotoxic effects of CDDP or Taxol were reduced in cells expressing
low amounts of iNOS (Fig. 3B). As reported previously (see refs. 5, 6), iNOS overexpression alone already resulted in enhanced apoptosis, which was further increased upon drug treatment (Fig. 3B).

**iNOS-induced cytoprotection requires activation of the PI3K/Akt pathway.** Because survivin up-regulation was reported to require PI3K/Akt activation in endothelial cells (23), we treated OVCAR-3 cells ectopically expressing low amounts of iNOS (0.2 μg pc4-iNOS–transfected cells), with the PI3K/Akt-inhibitor LY294002, and challenged them with cytotoxic drugs. PI3K/Akt inhibition resulted in reduced survivin expression (Fig. 3D), blocked up-regulation of phosphorylated Akt (data not shown), and enhanced drug-induced PCD (Fig. 3C). These data indicate that the PI3K/Akt pathway is involved in NO-mediated cytoprotection. Similar results were obtained in SKOV-3 cells (data not shown).

### Inhibition of iNOS and down-regulation of survivin cooperatively sensitize OCCs to drug induced apoptosis.

Given the cytoprotective effect of the NO/survivin-axis, we tested whether the pharmacologic inhibition of iNOS combined with the RNAi-mediated ablation of survivin may be applicable as a strategy to enforce PCD in OCCs. OVCAR-3 cells engineered to stably express low amounts of iNOS were transfected with survivin-siRNA or a scrambled control, and treated with the specific iNOS inhibitor 1400W. Subsequently, cells were challenged with cytotoxic drugs, and apoptosis was quantitated by measuring caspase-3 activity. The inhibition of iNOS enhanced Taxol/CDDP-induced cell death, which was significantly increased by the RNAi-mediated ablation of survivin (Fig. 4A). RNAi-/1400W–induced down-regulation of survivin levels were confirmed by immunoblot (Fig. 4B). Of note, in contrast to the cytotoxic effects induced by high doses of NO donors in HUVECs as well as in OCCs (Fig. 1B), treatment with 1400W did not affect proliferation of HUVECs (Fig. 4C).

**iNOS and survivin expression correlate in EOC patients.** To investigate the association between iNOS/survivin levels and clinical response, and to test the results obtained from our preclinical models in the clinical setting, we determined survivin/iNOS expression in EOC patients without residual disease by immunohistochemistry (IHC; Supplementary Table S1 for patient characteristics, and Supplementary Table S2 for summarized results).

Before IHC staining, we further controlled the specificity of the Abs used in our study. Therefore, iNOS and survivin were ectopically expressed in OVCAR-3 cells, and the proteins could be specifically detected in the cytoplasm (Fig. 5A). Of note, we rejected several commercially available α-iNOS Abs due to their high background staining (data not shown). To systematically analyze the protein levels in patient samples, we subsequently performed IHC of TMAs (Supplementary Table S2 for summarized results), which revealed low levels of the cytoplasmic iNOS protein in the majority of tumor biopsies (Fig. 5B). In contrast, survivin was readily detected in the cytoplasm and the nucleus of tumor cells, whereas the surrounding tissue was mostly negative (Fig. 5B and C). Because we observed a stimulation of survivin levels upon low iNOS expression in OCCs (Fig. 3), we examined the expression of the respective proteins in consecutive tumor sections. Representative examples illustrate that the low iNOS levels detectable in the majority of tumors (74%) correlated with enhanced survivin levels (Fig. 5C). For the computation of correlation coefficients, the noncategorized values were used. This analysis further confirmed that iNOS expression was significantly associated (rho, 0.39; \( P = 0.009 \)) with cytoplasmic survivin levels (IRScytl).

Correlation of iNOS and survivin levels with patient survival. We further analyzed the role of iNOS and survivin in predicting survival in completely debulked patients. Kaplan-Meier curves revealed an

![Figure 4](image-url)

**Figure 4.** iNOS inhibition and survivin depletion cooperatively sensitize OCCs to drug-induced apoptosis. Indicated \( P \) values were calculated using the two-sided Student's \( t \) test. A, OVCAR-3 iNOS-expressing cells were transfected with survivin-siRNA or a scrambled control. Twenty-four hours later, cells were treated with the iNOS inhibitor 1400W (50 μmol/L), and 16 h later, challenged with Taxol/CDDP. Measurement of caspase-3 activity by colorimetric assay was performed 24 h later. Caspase-3 activity in untreated cells transfected with the scrambled control was set to 1. Columns, mean from three independent experiments; bars, SD. B, immunoblot analysis of lysates from cells treated as described above using α-survivin Abs confirmed RNAi-/1400W–induced down-regulation of survivin. Reprobing with α-actin Abs served as loading control. C, high NO concentrations inhibit cell growth, whereas treatment with 1400W (50 μmol/L) does not significantly affect proliferation of HUVECs. HUVECs were incubated with the indicated concentrations of SNAP/SNP or the specific iNOS inhibitor 1400W, and proliferation was analyzed by the MTT assay. MTT-activity in untreated cells was set to 1. Columns, mean from three independent experiments; bars, SD.
improved PFS for patients with iNOS-negative tumors (log-rank test, \( P = 0.0414 \)), whereas iNOS expression was associated with poor prognosis (Fig. 6A). Also, Kaplan-Meier analysis indicated an improved PFS for patients with high nuclear and low cytoplasmic survivin (log-rank test, \( P = 0.0552 \) and \( P = 0.3836 \)), although the latter did not reach statistical significance. As a control, FIGO-stages (I–II versus III–IV) were highly significant associated with the risk of death from disease (log-rank test, \( P < 0.01 \); Fig. 6D).

Discussion

Molecular profiling studies (15, 24) and retrospective analyses of patient cohorts (12, 25) identified survivin as a “risk factor” for cancer progression and poor prognosis in ovarian carcinomas. Thus, the aim of the present study was to identify molecular pathways involved in the deregulated expression of survivin in EOCs as the basis for novel potential intervention strategies.

Here, we provide evidence that modulation of survivin by the small messenger NO contributes to the resistance of ovarian cancer cells against drug-induced apoptosis. The dual role of NO signaling and iNOS expression may both promote or inhibit tumor progression, depending on various conditions such as the genetic make-up of the cells, the local NO concentration, as well as the presence of other regulators (see refs. 5, 6).

One explanation for the double-edged effects of NO seems to be that high NO levels (in the \( \mu \text{mol/L} \) range) induce cytotoxic effects in tumor as well as in nonmalignant cells, e.g., HUVECs (refs. 5, 6, 26, 27, and references within). This assumption is further supported by our results showing that high-doses of chemical NO donors (SNAP, 0.5 mmol/L; SNP, 1 mmol/L) or strong overexpression of iNOS triggered apoptosis in vitro. However, various variables such as the microenvironment and/or the presence of other reactive oxygen species may influence these effects in vivo (see refs. 5, 6). Our results now indicate that survivin plays a critical role in NO-induced PCD because we observed down-regulation of survivin upon strong overexpression of iNOS or treatment with high-doses of SNP/SNAP. Also, NO-induced apoptosis was ameliorated upon ectopic survivin expression in OCCs. We further show that high NO levels inhibit survivin expression via the p38MAPK-dependent pathway. High NO concentrations stimulated phosphorylation of p38MAPK, and the NO-induced reduction of survivin levels was reversed by the p38MAPK inhibitor SB203580. Due to the different half-life of the applied NO donors (SNAP \( t_{1/2} \sim 5 \) hours; SNP \( t_{1/2} \sim 15 \) minutes), the proapoptotic effect of NO seems to be executed via a rapid activation of p38MAPK, leading to a irreversible cascade of molecular events ultimately culminating in cell death. p38MAPK has been associated with apoptosis in response to various cellular stresses, including NO-induced, caspase-3-associated apoptosis in other tumor models and macrophages (details in refs. 5, 26). Besides p38MAPK, other members of the MAP kinase family such as extracellular signal-regulated kinase or c-Jun NH2-terminal kinase have been implicated in NO-mediated apoptosis (5, 6). The detailed biochemical mechanism how NO directly or indirectly affects these kinases and which other kinases are also capable of modulating the NO/survivin-axis in OCCs remains to be investigated.

Importantly, we found that low concentrations of NO protect OCCs against drug-induced apoptosis by stimulating survivin expression. Several studies reported moderately low levels of iNOS activity in a variety of solid tumors, stimulating the idea that low NO concentrations may foster tumor promotion and disease progression. NO signaling was implied in activating various antiapoptotic pathways (28), involving bcl-2, p53 and/or direct inactivation of caspases by S-nitrosation (see refs. 5, 6). We here provide several lines of evidence strongly implicating that survivin acts as a major cytoprotective factor in EOCs. For one, ectopic expression of low iNOS levels as well as low concentrations of chemical NO donors enhanced survivin expression, which correlated with chemoresistance against Taxol- and cisplatin-induced...
cell death. Second, RNAi-mediated depletion of survivin significantly counteracted the cytoprotective effects of NO signaling and sensitized OCCs against drug-induced PCD. We show that NO induced phosphorylation of PI3K/Akt and that induction of survivin was reversed by the pharmacologic inhibition of PI3K/Akt, resulting in enhanced drug-induced apoptosis. Hence, NO-induced survivin expression downstream of the PI3K/Akt pathway represents an essential drug-resistance mechanism for EOC. Our findings are in agreement with previous reports from ovarian cancer (29) and other tumor cell models demonstrating that activation of the PI3K/Akt pathway by several stimuli including NO can significantly enhance resistance of tumor and tumor endothelial cells against chemotherapeutic regimens (refs. 6, 23, 30, and references within). Also, our results provide a molecular rationale for the enhancing effects of LY294002 on paclitaxel in reducing tumor burden in ovarian cancer mouse models (29).

The cytoprotective role of the iNOS/survivin pathway we uncovered in OCCs models also seems to be of clinical relevance. In our patient collective of serous EOC patients with macroscopically complete tumor resection, we found that the majority of tumors express low iNOS levels, which significantly correlates with enhanced survivin expression. The potential disease relevance of this correlation is further underlined by the Kaplan-Meier survival analysis, in which absence of iNOS-expression was a significant marker for better PFS. iNOS expression has also been suggested as an independent prognostic marker for poor survival in EOC (7). Several studies in different malignancies also indicate that iNOS positivity correlated with poor prognosis (6), although other studies propose that iNOS expression predicts better prognosis, or showed no relation to disease outcome (referenced in refs. 5, 31). Our results now provide a potential molecular rationale for these “inconsistencies,” and underline the necessity to accurately evaluate iNOS as well as survivin expression. Standardization of staining and scoring methods will be mandatory to evaluate and compare the prognostic relevance of the iNOS/survivin-axis in future comprehensive studies.

Although survivin expression has been convincingly linked to disease progression and therapy resistance in several malignancies (referenced in refs. 3, 4, 9), its role in ovarian cancer seems to be less clear (discussed in ref. 10). Our results are supported by other studies (see ref. 3), although Zaffaroni and colleagues (12) proposed that survivin was associated with resistance to the microtubule-targeting agent paclitaxel but unrelated to cisplatin responsiveness. However, we and others showed a cytoprotective function of survivin also against platin-based therapeutics (see refs. 3, 4, 9; this study). Hence, the molecular mechanism explaining this observation awaits further clarification. As previously shown for several malignancies (see refs. 4, 13), our analysis revealed that nuclear survivin, which seems to be impaired in its cytoprotective functions, is associated with favorable PFS also in EOC. A similar result was observed for metastatic EOC (25), although Ferrandina

![Figure 6](image_url)

Figure 6. Kaplan-Meier curves for PFS time in EOC patients by expression of the indicated proteins. Patients were dichotomized according to the IRS of the indicated proteins or FIGO stages. A, absence of INOS expression is a significant favorable predictor for PFS. B, predominantly nuclear survivin and (C) low cytoplasmic survivin levels are also indicators for prolonged PFS. D, FIGO-stages (I–II versus III–IV) are highly significant associated with PFS.

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