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Inhibition of Epithelial-to-Mesenchymal Transition (EMT) in Cancer by Nitric Oxide: Pivotal Roles of Nitrosylation of NF- κ B, YY1 and Snail

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Abstract

Treatment of cancer cell lines with high levels of nitric oxide (NO) via NO donors, such as DETANONOate, inhibits cell growth and survival pathways and sensitizes resistant tumor cells to apoptosis by chemoimmunotherapeutic drugs. In addition, we recently have reported that NO also inhibits the epithelial-to-mesenchymal transition (EMT) phenotype in metastatic cancer cell lines via dysregulation of the nuclear factor (NF)- κ B/Snail/Yin Yang 1 (YY1)/Raf kinase inhibitor protein circuitry. The mechanism underlying NO-mediated dysregulation of this circuit was investigated, namely, NO-mediated inhibition of the activity of the transcription factors NF- κ B, Snail, and YY1. We hypothesized that one mechanism of NO-mediated inhibition may invoke the NO-induced S-nitrosylation of these transcription factors. We demonstrate in metastatic and EMT⁺ human prostate carcinoma cell lines that treatment with NO results in the S-nitrosylation of NF- κ B (p50), Snail, and YY1 and inhibits their activities, resulting in the reversal of the EMT phenotype into a mesenchymal-to-epithelial transition phenotype. These findings suggest that NO donors may be potential therapeutic agents in both the reversal of resistance and the inhibition of EMT and metastasis.

Keywords

nitric oxide; epithelial-to-mesenchymal transition (EMT); Yin Yang 1; Raf kinase inhibitor protein; metastasis

I. INTRODUCTION

Cancer is a disease that is characterized by uncontrolled cell growth and proliferation resulting from genetic and epigenetic alterations.¹ Cancer also manifests itself to invasion and metastasis at different sites.² Cancer consists of a large number of diseases affecting many organs, and each disease has common as well as unique biochemical, molecular, and genetic features. The tumor initially is localized and has a good prognostic outcome through various treatment modalities, including surgery, radiotherapy, chemotherapy, hormonal therapy, and immunotherapy. However, often after treatment, the tumor recurs at the primary site and metastasizes, and such metastases are consistently resistant to conventional therapies.³ Resistance is invariably the result of various strategies of tumor cell acquisition

that inhibit the cytotoxic/apoptotic pathways responsive to cytotoxic therapies. Such strategies also have been implicated in the pathogenesis and progression of tumors as well as their metastatic behaviors.^{4–6}

In metastasis, the tumor cells invade the basement membrane and the circulatory system, extravasate through the endothelial lining of the blood vessels, and form colonies at different tissue sites. The migration of the epithelial layer into the mesenchymal layer before reaching the basement membrane is known as epithelial-to-mesenchymal transition (EMT).⁷

EMT is induced via alterations in gene expression of the primary tumor cells and normally via extracellular growth factors. Phenotypic alterations of EMT include loss of cell adhesion, basal apical polarity, and the gain of motility and spindle cell shape.⁸ In addition to endowing cancer cells with the invasive phenotype, EMT also helps cancer cells to evade apoptosis and acquire resistance to both immuno- and chemotherapeutic drugs.^{8,9} Tumor cells that have undergone EMT exhibit several molecular alterations that can be monitored for characterization of the EMT phenotype or of its loss. Hence, during EMT, the epithelial surface molecule, E-cadherin, is lost, whereas the expression of N-cadherin is highlighted, resulting in a cadherin switch.¹⁰ For β -catenin, the intracellular localization of this protein is associated with EMT because it is localized on the cell membrane in epithelial cells. In mesenchymal cells, β -catenin is translocated into the nucleus, where it acts as a co-activator of transcription factors associated with EMT.^{11,12} Other markers of EMT include the extracellular proteins such as fibronectin and laminin, cytoskeletal markers such as vimentin, as well as transcription factors such as Snail and Twist.¹³ In EMT, the epithelial markers E-cadherin, α -catenin, and γ -catenin are repressed, whereas the mesenchymal markers vimentin, fibronectin, and N-cadherin are induced. In addition, certain microRNAs have been proposed to be key markers of EMT.¹⁴

We recently have reported the dysregulated nuclear-factor (NF)- κ B/Snail/Yin Yang (YY) 1 /Raf kinase inhibitor protein (RKIP)/phosphatase and tensin homologue (PTEN) circuitry in cancer cells, and this circuit has been shown to play a pivotal role in the EMT phenotype.^{9,15–17}

The role of nitric oxide (NO) in cancer has been the subject of controversy, with reports suggesting either its anti- or proneoplastic activity. This biphasic role of NO in oncology seems to be dependent on the microenvironment, the responsiveness of the tumor type, the redox state of the chemical reaction, as well as the final concentration and the duration of its intracellular exposure.^{18–20} This controversy might have been resolved, in large part, by the demonstration that low concentrations of NO act as an enhancer of cancer cell survival and proliferation and protect the cancer cells from apoptosis. However, high concentrations of NO exhibit, for example, direct cytotoxic effects and gene mutations, all of which contribute to tumor regression.^{21,22}

We have reported that treatment of drug-resistant and metastatic human cancer cell lines with high levels of NO donors (e.g., DETANONOate) resulted in both the sensitization of drug-resistant tumor cells to apoptosis by chemoimmunocytotoxic drugs and the inhibition of the EMT phenotype (Fig. 1).⁹ On the basis of these findings, we hypothesized that NO-induced inhibition of EMT may be the result of NO-mediated inhibition of NF- κ B, YY1, and Snail activities via S-nitrosylation. Here we review briefly the effect of NO on EMT via its effects on each of the factors in the circuitry (NF- κ B, Snail, YY1, and RKIP) and the demonstration that each of these factors modified by NO is involved directly in the regulation of EMT.

II. NO-MEDIATED INTERFERENCE IN THE NF- κ B/SNAIL/YY1/RKIP CIRCUIT AND THE RESULTANT INHIBITION OF EMT

We have reported that treatment of EMT⁺ metastatic human carcinoma cell lines with DETANONOate inhibits the EMT⁺ phenotype through downregulation of the mesenchymal markers vimentin and fibronectin and induction of the epithelial marker E-cadherin, along with the inhibition of invasiveness. These effects were accompanied by the inhibition of NF- κ B, Snail, and YY1 and the induction of RKIP.

II. A. NO-Mediated Inhibition of NF- κ B Activity via S-nitrosylation of p50 and Inhibition of EMT

It has been reported that activation of NF- κ B regulates positively EMT and metastasis.^{23–25} Treatment of EMT⁺ prostate carcinoma cell lines with DETANONOate inhibited NF- κ B DNA-binding activity as assessed by electrophoretic mobility shift assay. Furthermore, inhibition of NF- κ B by the specific NF- κ B inhibitor, DHMEQ, also inhibited EMT.²⁶ In addition, treatment with DETANONOate resulted in the S-nitrosylation of p50²⁷ (Huerta-Yepez et al, unpublished data). The S-nitrosylation of p50 inhibited the nuclear translocation of NF- κ B into the nucleus and inhibited the transcription and expression of its target genes such as Snail and YY1 (Fig. 2).

II. B. NO-Mediated Inhibition of Snail Activity via S-nitrosylation, Leading to Inhibition of EMT

Snail is a member of the Snail superfamily of zinc finger transcription factors. It has many functions²⁸ and plays a pivotal role in the acquisition of migratory and invasive properties and triggers EMT by repressing the expression of E-cadherin.²⁹ The transcription of Snail is under the regulation of NF- κ B and is a transcription repressor of the metastatic suppressor gene product E-cadherin and RKIP.^{30,31} Snail silencing reverses the EMT phenotype, whereas its overexpression induces EMT.^{32,33}

We also recently have reported that treatment of cancer cells with NO results in the S-nitrosylation of Snail.²⁶ Therefore, we suggest that NO-mediated inhibition of Snail is the result of both the inhibition of its expression via the inhibition of NF- κ B and directly via NO-mediated S-nitrosylation. Both the expression and the activity of Snail modified by NO are involved in the inhibition of EMT (Fig. 3).

II. C. NO-Mediated Inhibition of YY1 via S-nitrosylation, Leading to the Inhibition of EMT

YY1 is a multifunctional, DNA-binding protein or associates with other transcription factors and functions as either an activator or a repressor.³⁴ The transcription factor YY1 is regulated transcriptionally, in part by NF- κ B and YY1; in turn, YY1 regulates the transcription of Snail.^{35,36} The role of YY1 in the regulation of EMT via the dysregulated NF- κ B/Snail/YY1/RKIP/PTEN circuitry has been reviewed recently by our group.⁹ We also have reported that NO S-nitrosylates YY1 and inhibits its DNA-binding activity.²⁷ Several lines of evidence suggest the direct role of YY1 in the regulation of EMT. Treatment of hepatic cells with YY1 siRNA reduced the invasive activity in the cells.³⁷ Our preliminary findings show that silencing YY1 by YY1 siRNA decreases the expression of the mesenchymal markers vimentin and fibronectin and induces the expression of the epithelial marker E-cadherin along with inhibition of Snail and the induction of RKIP. de Nigris et al³⁸ reported that overexpression of the YY1 protein was associated with malignancy and deletion of YY1 by siRNA metastatic growth. These findings suggest that NO-mediated inhibition of Snail expression and activity is a result of both the inhibition of its transcription

by NO-mediated inhibition of NF- κ B and its direct inhibition of activity by S-nitrosylation (Fig. 4).

II. D. NO-Mediated Induction of RKIP and Its Role in the Inhibition of EMT

In contrast to NO-mediated inhibition of NF- κ B, Snail, and YY1, NO treatment has an inducing effect on RKIP expression. RKIP is a member of the phosphatidyl-ethanolamine protein family present in cytoplasm.³⁹ Yeung et al^{40,41} have reported that RKIP inhibits the activity of both the Raf-1/MEK/extracellular signal-regulated kinase and NF- κ B pathways. The expression of RKIP is downregulated significantly in several malignancies and is almost absent in several metastatic tumors. Overexpression of RKIP inhibits metastasis in experimental animal model systems.^{42,43} Hence, RKIP was identified as a metastatic suppressor gene product.⁴² Snail represses RKIP transcription.³⁰ The direct role of RKIP in the regulation of EMT was corroborated in an experiment whereby ectopic expression of RKIP inhibited expression of Snail and the mesenchymal gene markers vimentin and fibronectin and reversed the EMT phenotype and inhibited cell migration and invasion (Fig. 5).²⁶

III. *IN VIVO* NO-MEDIATED INHIBITION OF THE EMT PHENOTYPE IN A METASTATIC HUMAN TUMOR XENOGRAFT

We have investigated the effect of *in vivo* treatment of mice bearing EMT⁺ PC3 human prostate carcinoma cells on the EMT phenotype. Tumor-bearing mice were treated intratumorally with DETANONOate, and samples then were analyzed by immunohistochemistry for several EMT biomarkers. The findings revealed that after treatment with DETANONOate, the expression of the mesenchymal markers fibronectin and vimentin were along with the expression of Snail. In contrast, the expression of both E-cadherin and RKIP were significantly induced (Huerta-Yepez et al, unpublished data). These findings corroborate the *in vitro* findings and validate the role of NO in the inhibition of the EMT phenotype in tumor-bearing mice.

IV. CONCLUSIONS

The findings reported here show that treatment of EMT⁺ human cancer cell lines with the NO donor DETANONOate inhibits EMT via modification of the dysregulated NF- κ B/Snail/YY1/RKIP loop, which has been shown to regulate EMT. Each of the gene products in the loop directly regulates EMT by either silencing it or following ectopic expression in EMT⁺ and EMT⁻ tumor cells, respectively. The NO-mediated inhibition of EMT is a result, in part, of the S-nitrosylation of the transcription factors NF- κ B, Snail, and YY1. The S-nitrosylation of these factors inhibits their DNA-binding activities. The inhibition of these factors resulted in the induction of RKIP expression, which is under its transcriptional regulation, in part, by Snail. The *in vitro* findings of NO-mediated inhibition of EMT were corroborated *in vivo* in mice bearing EMT⁺ tumor xenografts. Treatment with the NO donor DETANONOate inhibited *in vivo* the EMT phenotype. Together, these findings suggest potential therapeutic efficacy of NO donors in the inhibition of EMT and metastasis as well as their roles in sensitizing drug-resistant tumor cells to apoptosis by cytotoxic drugs.

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ABBREVIATIONS

DETANONOate	(Z)-1-[2-(2-aminoethyl)-N-(2-ammonio-ethyl) amino] diazen-1-ium-1,2-diolate
NF-κB	nuclear factor κB
NO	nitric oxide
RKIP	Raf kinase inhibitor protein
YY1	Yin Yang 1

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FIGURE 1.

General schematic diagram demonstrating the constitutively activated nuclear factor(NF)- κ B/Snail/Yin Yang (YY) 1/RKIP circuitry in tumor cells and their modification by nitric oxide (NO). In tumor cells, the NF- κ B pathway is activated constitutively. It has been reported that NF- κ B regulates the transcription and expression of both Snail and YY1 and that YY1 also regulates Snail transcription. Snail represses RKIP, which has been reported to inhibit NF- κ B. The overexpression of NF- κ B, Snail, and YY1 and the underexpression of RKIP have been shown to regulate the epithelial-to-mesenchymal transition (EMT) phenotype and metastasis. Treatment with the NO donor DETANONOate results in the inhibition of NF- κ B, Snail, and YY1 and the induction of RKIP expressions. This results in the reversal of the repression of E-cadherin by Snail and upregulation of E-cadherin. In addition to NO-induced inhibition of NF- κ B and its target genes, it also inhibits the DNA-binding activities of NF- κ B, Snail, and YY1 via S-nitrosylation. Together, these various modifications by NO result in the inhibition of EMT and metastasis.

FIGURE 2.

The direct role of nitric oxide (NO)–induced inhibition of nuclear factor (NF)–κB in the inhibition of the epithelial-to-mesenchymal transition (EMT). Treatment of cells with NO results in the inhibition of NF-κB expression and activity via S-nitrosylation of p50 and p65. This inhibition results in the inhibition of NF-κB target genes Yin Yang (YY) 1 and Snail expressions as well as their activities via nitrosylation by NO. The inhibition of Snail results in the de-repression of RKIP and its induction; in turn, RKIP amplifies the inhibitory activity of NF-κB. Together, NO treatment results in the inhibition of EMT.

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FIGURE 4.

The direct role of nitric oxide (NO)–induced inhibition of Yin Yang (YY) 1 in the inhibition of the epithelial-to-mesenchymal transition (EMT). Treatment with NO inhibits YY1 activity via S-nitrosylation and its expression via inhibition of nuclear factor (NF)-κB. NO-mediated inhibition of YY1, Snail, and NF-κB results in the induction of RKIP. Overexpression of YY1 induces EMT and its knockdown inhibits EMT. Together, NO-induced inhibition of YY1 results in the inhibition of EMT.

FIGURE 5.

Nitric oxide (NO)-induced upregulation of RKIP in the inhibition of the epithelial-to-mesenchymal transition (EMT). Treatment with NO results in the inhibition of the transcription repressor Snail, which results in the induction of RKIP expression, a metastasis suppressor gene product. Upregulation of RKIP amplifies the inhibition of the nuclear factor (NF)-κB/Snail/Yin Yang (YY) 1 loop. Overexpression of RKIP results in the inhibition of EMT and its knockdown results in the induction of EMT. Together, NO-induced upregulation of RKIP results in the inhibition of EMT.