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Nitric Oxide Donor Exisulind is an Effective Inhibitor of Murine Photocarcinogenesis

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Abstract

NO-releasing nonsteroidal anti-inflammatory drugs (NO-NSAIDs) have been shown to have anti-inflammatory, anti-proliferative and apoptosis-inducing effects in tumor cells. Here we have investigated the effects of NO-exisulind on the growth of UVB-induced skin tumor development in a murine model. We found that the topical treatment with NO-exisulind significantly reduced UVB-induced tumors in SKH-1 hairless mice. The tumors/tumor bearing mouse, the number of tumors/mouse and tumor volume/mouse decreased significantly ($p < 0.05$) as compared to vehicle-treated and UVB-irradiated positive controls. Consistently, NO-exisulind-treated animals showed reduced expression of proliferation markers such as PCNA and cyclin D1. These mice also manifested increased expression of pro-apoptotic Bax and decreased expression of anti-apoptotic Bcl2 with an increase in the number of TUNEL-positive cells in tumors. We also investigated whether NO-exisulind-treated tumors are less invasive and progress less efficiently from benign to malignant carcinomas. For this, tumors were stained for various epithelial-mesenchymal transition (EMT) markers. NO-exisulind decreased the expression of mesenchymal markers such as Fibronectin, N-cadherin, SNAIL, Slug and Twist and enhanced the epithelial marker E-cadherin. Similarly, UVB-induced phosphorylation of Erk1/2 and p38 was decreased in NO-exisulind-treated animals. These data suggest that NO-exisulind reduces tumor growth and inhibits tumor progression by blocking proliferation, inducing apoptosis and reducing EMT.

Keywords

NSAIDs; NO-exisulind; photocarcinogenesis; skin; UVB

INTRODUCTION

Exisulind (sulindac sulfone) is a metabolite of the nonsteroidal anti-inflammatory drug (NSAID), sulindac. Sulindac is known to be metabolized to sulindac sulfide by a reversible reaction or to sulindac sulfone irreversibly. The sulfone metabolite lacks cyclooxygenase (COX) inhibitory activity whereas sulfide is the most active metabolite of sulindac (1,2). It is believed that non-target pharmacological activities of exisulind are important for diminishing tumor growth as it does not act through COX-dependent prostaglandin synthesis inhibition. In this regard, its ability to block proliferation through the inhibition of cyclin

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D1-dependent cell cycle progression and its ability to induce apoptosis are considered important. Sulindac was shown to be an effective inhibitor of solid tumor growth in various murine models (3,4).

NO-releasing NSAIDs (NO-NSAIDs) are a recently described class of NSAIDs which consist of traditional NSAIDs to which an NO moiety is covalently attached via a linker also called a spacer molecule (5). The rationale for the development of these NO-NSAIDs was to combine the effects of NSAIDs and NO in order to confer the protective effects of NO on the gastric mucosa while maintaining the desired COX-dependent anti-inflammatory activity. Based on this notion, NO-aspirin (NO-ASA), NO-sulindac and NO-ibuprofen among others have been synthesized and were found to possess chemopreventive properties similar to those associated with their parent compounds. Interestingly, under certain experimental conditions NO-NSAIDs were found to be more potent than their corresponding NSAID in reducing tumorigenesis in animal models (5, 6).

Here we show that NO-exisulind reduces UVB-induced tumorigenesis in SKH-1 hairless mice. The chemopreventive effects of NO-exisulind were related to its ability to block proliferation, induce apoptosis and reduce epithelial-mesenchymal transition (EMT) markers in tumor keratinocytes.

MATERIALS AND METHODS

NO-exisulind (Fisher Biosciences, Germantown, MA) was stored at room temperature. NO-exisulind ($C_{24}H_{24}FNO_7S$) is a yellow crystalline powder which is soluble in chloroform (Fig. 1). Animals were maintained under standard conditions of a 12 h dark/12 h light cycle, a temperature of $24 \pm 2^\circ C$ and relative humidity of $50 \pm 10\%$. The animal protocol used in this study was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Alabama at Birmingham. Following irradiation for 30 weeks, the experiment was terminated and all mice were euthanized as per IACUC recommendations

UV light source and irradiation of animals

To irradiate the experimental animals, we used a UV Irradiation Unit (Daavlin Co., Bryan, OH) equipped with an electronic controller to regulate dosage of irradiated UVB. The UVB source consisted of six Philips Ultraviolet B TL 40W/12RS lamps. Further, we used a Kodacel cellulose film (Kodacel TA401 / 407) to eliminate any UVC.

Experimental design

For this experiment, 6-8 week old SKH-1 hairless mice were divided into three groups of fifteen animals each. Group-I animals received only vehicle (acetone) and served as an age-matched negative control. Group-II and III mice were irradiated with UVB ($180\text{mJ}/\text{cm}^2$; twice/week) for 30 weeks. In addition to UVB irradiation, group II and III mice received twice weekly topical treatments (30 min prior to UVB irradiation) with either vehicle (acetone) or NO-exisulind (5 mg/mouse dissolved in $200\mu\text{l}$ acetone) respectively.

Measurement of tumor size

Tumor numbers were recorded and the dimension of each tumor was measured in terms of tumor length, width, and height by using Vernier Calipers. The formula, tumor volume = $\pi \times \text{radius}^2 \times \text{height} / 3$ was used to calculate tumor volume. Data were presented as mean $\pm$ SE and plotted as a function of weeks on test.

Preparation of tissue lysate for Western blot analysis

Skin/tumor tissue samples were washed with ice cold 1×PBS and lysed in cold lysis buffer (50 mM Tris pH, 1% Triton X 100, 0.25% NaF, 10mM β-glycerophosphate, 1mM EDTA, 5mM sodium pyrophosphate, 0.5 mM Na₃VO₄, 10mM DTT, 1% PMSF and protease inhibitors). For immunoblotting, proteins (60-80μg) were resolved on 10-15% SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking the non-specific binding sites, the membrane was incubated with primary antibodies (Supplementary Table 1) at 4°C overnight. The membrane was then incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies and the immunoreactive bands were visualized using an enhanced chemiluminescence reagent (Pierce, Rockford, IL, USA). Membranes were stripped and reprobbed with anti-β-actin antibody to verify equal protein loading.

Densitometry

The numerical values shown above each Western blot are mean values from three individual bands in one group. In each case the values for the negative control group used was taken as “1” and comparison was made with densitometry values obtained from vehicle or NO-exisulind-treatment groups. The comparative data are presented as fold-change after treatment as compared to negative controls.

Histologic evaluation of tumor burden

Paraffin sections (6 μm) were used for the histologic determination of tumors. The sections from each group of mice were stained with H&E and examined for tumor histology.

Immunohistochemistry and Immunofluorescence staining

Paraffin sections (6 μm) were deparaffinized and rehydrated through xylene and graded alcohol. Slides were placed in antigen unmasking solution (Vector laboratories) and microwaved until boiling. Peroxidase blocking was performed by using 3% H₂O₂ in 1×PBS for 20 minutes. The nonspecific sites were blocked in 2% BSA for 45 min at ambient temperature, followed by overnight incubation at 4°C in primary antibody. Samples treated with blocking serum (2% BSA) instead of primary antibody served as negative controls. For immunohistochemistry, sections were washed and incubated with biotinylated secondary antibody and then with HRP conjugated streptavidin. Color reaction was observed using 3, 3'-diaminobenzidine. Sections were counterstained with Harris hematoxylin (Sigma-Aldrich), dehydrated through butanol-xylene and mounted using Permount (Fisher Scientific). Immunofluorescence staining was performed using Alexa Fluor 594 (Invitrogen, Carlsbad, CA, USA), Dylight 488 (Pierce) or Fluorescein (Pierce)-coupled secondary antibody. Sections were mounted with Vectashield Mounting Medium with DAPI (H-1200; Vector Laboratories).

Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) analysis was performed using a kit from Roche Applied Science (Cat. no.1684795) according to manufacturer's guidelines.

Statistical analysis

Statistical analysis was performed using Microsoft Excel software. The significance between two groups was determined using two-tailed Student's t-test with p<0.05 considered as significant.

RESULTS

Treatment of mice with NO-exisulind reduces UVB-induced photo- carcinogenesis

Topical application of NO-exisulind resulted in a significant reduction in photocarcinogenesis when assessed in terms of percent of mice with tumors and tumor multiplicity compared with UVB-irradiated positive control animals as shown in Fig. 2. Over the 30 week duration of this experiment, a total of 10.53 ± 1.72 tumors/tumor bearing mouse were recorded in the UVB-irradiated group while only 5 ± 0.80 tumors were recorded in the NO-exisulind-treated animals (Fig. 2A & B). NO-exisulind treatment also resulted in significant tumor reduction and delay of tumor incidence when compared at early time points with UVB alone positive controls. However, tumor incidence at the 28th week was 80% and increased to 100% by the 30th week in both NO-exisulind treated and UVB-alone irradiated treatment groups (Fig. 2C). Similarly, NO-exisulind significantly reduced tumor volume when assessed in terms of mean tumor volume/mouse ($p < 0.05$) as shown in Fig. 2D. Application of NO-exisulind also slightly increased the latency period of tumor induction.

To compare mice in terms of tumor numbers and to demonstrate the difference in tumor burden following NO-exisulind treatment, we divided them into groups consisting of 0-5, 6-10 and >10 tumors/mouse. NO-exisulind treatment resulted in a decrease in the high tumor number/tumor bearing mouse groups. Thus in the NO-exisulind treatment group, 60% of mice were found to have tumor numbers ranging from 0-5 while the remaining 40% of mice were found to have 6-10 tumors/mouse. No animals in the NO-exisulind treatment group were found to have >ten tumors/mouse as shown in Fig. 3A. In the UVB alone group only 20% of mice exhibited tumor numbers ranging from 0-5 while 40% had between 6-10 tumors and the remaining 40% had >10 tumors (Fig. 3A).

NO-exisulind treatment reduces aggressive tumor-phenotype and markers of proliferation

As shown in Fig. 3B, NO-exisulind treatment significantly reduced ($p < 0.05$) the induction of aggressive SCCs. Analysis of H&E stained tumor sections from vehicle-treated and UVB-irradiated positive controls revealed that some of these tumors were poorly differentiated SCCs with hyperchromatic pleomorphic nuclei in which there was considerable mitotic activity. Interestingly, a majority of the SCCs in mice treated with NO-exisulind displayed a less aggressive and more differentiated tumor phenotype characterized by the presence of small rounded keratin pearls (Fig. 4A).

We also examined the effects of NO-exisulind on the biomarkers of cell proliferation by assessing the protein expression of proliferation cell nuclear antigen (PCNA) and cyclin D1 (Figure 4A & B). UVB-induced PCNA expression in tumors was substantially reduced in NO-exisulind-treated mice. Similarly, the expression of cyclin D1 was also found to be less prominent in the NO-exisulind group as compared to the UVB alone treatment group. Other cell cycle regulatory proteins such as cyclins A/B1 and E2F were also reduced (Fig. 4C).

NO-exisulind treatment leads to induction of apoptosis with a concomitant downregulation of antiapoptotic Bcl2 and upregulation of proapoptotic Bax proteins

The number of TUNEL-positive cells was greater in UVB-induced tumors treated with NO-exisulind as compared to UVB-induced vehicle-treated tumors (Fig. 4D). Increased expression of Bax is known to accelerate apoptotic cell death while Bcl2 enhances cell survival by inhibiting apoptosis (7). Thus the ratio of Bax:Bcl2 determines death to survival signal following apoptotic stimuli. The Bax/Bcl2 ratio was significantly increased ($p < 0.05$) in the NO-exisulind treatment group, which suggests the possibility that NO-exisulind-mediated tumor cell death occurs by an intrinsic apoptosis mechanism.

NO-exisulind decreases phosphorylation-dependent activation of mitogen activated protein kinase (MAPK) regulatory proteins

UVB, possibly by augmenting oxidative stress, enhances phosphorylation of MAPK kinase proteins thereby transducing signaling following extracellular stimuli to regulate proliferation and inflammation (8). MAPK proteins such as Erk1/2 and p38 are thought to play an important role in cell proliferation, differentiation and apoptosis in response to various external signals and have been implicated in multistage skin carcinogenesis (9). Therefore, in this study we determined the effects of NO-exisulind on UVB-induced phosphorylation of ERK1/2 and p38 along with other markers of inflammation (e.g. iNOS and COX-2). While no significant effect of NO-exisulind was noted on COX-2 expression, iNOS levels were attenuated to the level of age-matched controls (Fig. 5A). Phosphorylation of ERK1/2 and p38 was induced by UVB as compared to age-matched controls by 3.37- and 9.33-fold respectively as ascertained by Western blot analysis. However, this induction was diminished following NO-exisulind treatment (Fig. 5B). Similar results were obtained from immunostaining data of p-Erk1/2 and p-p38 (Fig. 5C).

NO-exisulind treatment acts by altering UVB-induced EMT

To test whether the observed blockade in the progression of benign lesions to malignant SCCs by NO-exisulind is due to its effects on EMT, we stained tumors for epithelial marker protein E-cadherin and mesenchymal marker proteins Fibronectin, N-cadherin, SNAI, Slug and Twist. The alterations in the expression of these proteins are considered as critical regulators of EMT. As shown in Fig. 6, NO-exisulind treatment of UVB-induced tumors retained the expression of E-cadherin while simultaneously reducing Fibronectin, N-cadherin, Snail, Slug and Twist.

DISCUSSION

Our laboratory has an ongoing interest in investigating the role of NSAIDs as chemopreventive agents against NMSCs (10). Earlier we showed that UVB-induced skin neoplasm both in humans and in experimental animals manifest enhanced expression of COX-2 as well as COX-2-derived prostaglandin PGE₂ receptor expression (11-14). We also showed that NSAIDs such as sulindac and nimesulide reduced phototoxic and carcinogenic effects of UVB (15, 16). Sulindac sulfide, the most active metabolite of sulindac is concentrated in colonic epithelium at concentrations at least 20-fold higher than those seen in serum which range between 10-15 μ M (2). However, it is not known whether similar accumulation of this and other sulindac metabolites occurs in skin and other tissues. In addition, our laboratory has shown that NO-releasing agents afford protective effects against two stage initiation-promotion chemical carcinogenesis (17). Therefore, in this study an agent that has both NO-releasing properties and NSAID-related anti-inflammatory effects seems to be highly promising. Since we applied NO-exisulind topically, it is likely that local concentration in cutaneous keratinocytes may be very high. We therefore believe that the critical concentration of this NSAID required to manifest biological effects is locally achieved by its topical application. Topical NO-exisulind in this study exhibits a strong chemopreventive effect against UVB-induced murine skin carcinogenesis by eliminating rapidly proliferating tumors, particularly SCCs. These observations are consistent with the decrease in cell cycle regulatory proteins and proliferation marker PCNA in tumor cells. In addition, this NSAID has also been found to induce apoptosis thus providing a basis for the decrease in tumor volume. These effects appear to be regulated through diminution of the MAPK cascade, which has been implicated in the regulation of apoptosis as well as inflammation (18-20). Consistently, exisulind-induced apoptosis in human colon cancer cells has been shown to involve the inhibition of ERK1/2 (21, 22). These observations also suggest that most of the proliferation-inhibiting and apoptosis-inducing effects of NO-

exisulind may be due to the exisulind moiety of this molecule. The observed reduction in iNOS in UVB-induced tumors may be related to its effects on inflammatory cells, particularly phagocytes, although the role of iNOS in keratinocytes in mediating these effects cannot be ruled out (23). However, this remains to be ascertained. Similar anti-inflammatory and photoprotective effects were noted in our earlier studies (14, 15). Tumor invasion/metastasis is a complex process and involves a large number of proteins known to play an important role in embryonic development. In this regard, proteins related to the phenomenon known as EMT, a process in which cells undergo a developmental transition from epithelial to a motile mesenchymal phenotype by losing their cell-cell adhesion properties, is important in achieving an invasive tumor phenotype (24-26). Solid tumor progression requires EMT for tumor cell invasion and metastasis (24, 25). Although NMSCs rarely metastasize, they are highly locally invasive leading to tissue destruction and disfigurement (27). Therefore, chemopreventive agents that in addition to diminishing tumor burden have properties related to blocking tumor invasiveness are of great advantage. NO-exisulind in this study was found to possess both tumor burden and invasiveness diminishing properties.

The cadherins are among the important mediators of EMT, which involves calcium-dependent, transmembrane glycoproteins (26). A transcriptional factor, involved in downregulation of E-cadherin, twist, has been shown to play a critical role in tumor metastasis (28). It has been reported that epithelial marker, E-cadherin is downregulated and mesenchymal N-cadherin is upregulated in twist-induced cells (28). N-cadherin also leads to activation of MAPK-ERK signaling cascades (29). Snai1 and Snai2 (Slug) are considered as key regulators of EMT and important for tumor invasion (30-33). Recent studies showed that Snai1/2 are expressed in various epithelial carcinomas including those of breast, ovary and colon and are often associated with tumor invasion, metastasis and/or poor prognosis (31,32). In some cancers, Snai1 is found to be a determinant of tumor invasiveness while Snai2 is involved in the establishment of distal metastasis during the progression stages of murine carcinogenesis (33). Thus, high expression of these marker proteins in UVB-induced skin tumors suggests that these tumors may have the ability to invade and metastasize. Our data showing that NO-exisulind can block these effects suggest a possibility that this NSAID may act at late stages of tumorigenesis. In summary, these results indicate that NO-exisulind is a potent cutaneous cancer chemopreventive drug and has the capacity to block tumor progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.
Chemical structure of NO-exisulind

Fig. 2. Topical application of NO-exisulind inhibits UVB-induced skin tumor development in SKH-1 hairless mice

Data shown here were recorded on a weekly basis and summarized at the termination of the experiment at week 30 of UVB irradiation. Mice were divided into three groups, Group 1 - negative control (No tumor developed in this group and therefore data is not shown in this figure), Group 2 - vehicle-treated and UVB-irradiated positive control, and Group 3 - UVB-irradiated mice receiving NO-exisulind treatment. Vehicle (200 μ l acetone) or NO-exisulind treatment was administered at least thirty minutes prior to UVB (180 mJ/cm²) exposure. A) NO-exisulind inhibits UVB-induced tumor development represented as tumors/tumor bearing mouse, B) number of tumors/mouse, C) % tumor incidence, D) tumor volume/mouse represented as mean (mm³) $\pm$ SE. At least * $p < 0.05$ was considered significant when compared to the UVB (alone) treatment group at the termination of the experiment.

Fig. 3. NO-exisulind reduces tumor body burden and blocks SCC development

A) profile of tumor body burden in UVB alone and NO-exisulind+UVB groups, B) Number of SCCs following NO-exisulind treatment. Tumor number was represented as mean $\pm$ SE

Fig. 4. NO-exisulind blocks proliferation and induces apoptosis in UVB-induced tumors
Paraffin fixed tumor sections (6μm) were subjected to H&E staining and immunohistochemical analyses for expression of proliferation markers. NO-exisulind treatment resulted in a less aggressive tumor phenotype as evidenced by the presence of keratin pearls, a marker of well differentiated cutaneous SCC (Figure 4A and panel insets) and a reduction in the expression of proliferation markers such as PCNA, Cyclin A1 and Cyclin D1 (Figures 4B&C). Representative tumor micrographs are shown. Images were taken at 20X magnification. TUNEL staining showing apoptosed cells, and Western blotting showing the expression of pro-apoptotic Bax and anti-apoptotic Bcl2 (Figure 4D). Bax:Bcl2 ratio was calculated by using densitometric values of each individual band and expressed as mean±SE of **three** individual values. Significance between two groups was determined using Student's t-test.

Fig. 5. NO-exisulind inhibits UVB-induced phosphorylation of MAPK proteins

Paraffin sections from UVB-irradiated and NO-exisulind-treated mice were subjected to immunofluorescent staining of iNOS, p-ERK1/2 and p-p38. Expression of iNOS was attenuated in NO-exisulind-treated tumors as compared to UVB-irradiated tumors, whereas there is no significant effect on COX-2 expression (5A). The expression levels of p-ERK1/2 and p-p38 were reduced in NO-exisulind-treated mice compared to their vehicle-treated UVB-irradiated counterparts (5B). Representative immunofluorescence stained sections of tumor micrographs (selected from 5 individual replicates) are represented (5C). Images were taken at 20X and 40X magnifications. Tumor lysates were prepared to determine the phosphorylated and total protein levels of ERK1/2 and p38 using Western blot analysis.

Fig. 6. NO-exisulind reduces expression of mesenchymal markers

Topical treatment with NO-exisulind reduces UVB-induced epithelial mesenchymal protein expression. Epithelial protein (E-cadherin), mesenchymal related protein such as N-cadherin, Fibronectin and Slug, SNAI and Twist expression were analyzed by immunofluorescence staining. Briefly, 6 μ m paraffin fixed sections were subjected to various immunofluorescence staining and tumor micrographs were recorded at 10X magnifications.