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Original Research Article

Effect of dose-rate on nitric oxide-induced apoptosis in A375 human melanoma cells

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Abstract

Purpose: *To investigate the effect of dose-rate on nitric oxide (NO)-induced apoptosis in A375 human melanoma cells.*
Methods: *The A375 cells were exposed to NO at a steady-state concentration of 7 µM, similar to the level estimated to occur in vivo in inflamed tissues, and delivered by diffusion through silastic tubing. Trypan blue dye exclusion assay was used to assess cell proliferation and viability. Cell cycle was studied by propidium iodide (PI) staining while Annexin V-FITC and PI assays were used to evaluate apoptosis. Western blotting assay was carried out to determine protein levels of p53, Bax, DR4, DR5, Fas (CD95), Procaspase 8, Procaspase 9 and Procaspase 3.*
Results: *Viability of A375 cells was reduced by 22 % after 24 h leading to extensive apoptosis and cell cycle arrest following exposure to a cumulative dose of 3360 µM/min NO. Treatment with NO stimulated p53 and triggered mitochondrial apoptotic events by inducing conformational changes in Bax. Also, activation of caspase 9 and 3, DR4, DR5, Fas (CD95) and caspase 8 appeared to be mediated concurrently by death receptor processing and downstream caspases.*
Conclusion: *Nitric oxide (NO) induces programmed cell death, thus indicating that it could serve as an effective inhibitor to halt the progression of melanoma or as an enhancer to improve therapeutic strategies for treatment.*

Keywords: *Apoptosis, A375, Cell cycle arrest, Human melanoma, Nitric oxide*

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INTRODUCTION

Melanoma, which originates from pigment-producing melanocytes, stands out as the most aggressive and deadly form of skin cancer. It's widely recognized for its resistance to all existing cancer treatment methods [1,2]. Although melanoma accounts for only 4 % of all skin cancers, it is responsible for almost 80 % of skin cancer-related deaths [1,2]. Patients with visceral metastasis have a survival rate of < 20 % beyond 5 years and surgical excision are the only reliable means of control [1,2]. As a result of the rising incidence of melanoma and its resistance to treatment, there is a pressing need for new strategies in both prevention and treatment. Apoptosis, known as programmed cell death, exhibits a distinct set of morphological and biochemical features. Dysfunctions in this process contribute to the development of

numerous diseases [3,4].

Recent evidence suggests that nitric oxide (NO) is a powerful regulator of homeostasis, which may either prevent or induce apoptosis. These effects are influenced by both direct and indirect interactions, which may vary depending on dosage and specific cell type [3,5]. In some cell types, NO promotes apoptosis, whereas in others, it inhibits apoptosis [5]. For example, previous studies have extensively investigated NO-induced cellular stress, DNA damage, mutagenesis and apoptotic signaling pathways in human lymphoblastoid cells [4,6]. In contrast, other studies recently have shown that nitric oxide synthases (NOS) activity and inducible nitric oxide synthase (iNOS) expression in melanoma growth, invasiveness and metastasis, indicated that endogenous NO may serve as a survival factor in human melanoma cells [4].

There has been a lot of focus on developing diverse and promising experimental anticancer drugs that regulate apoptotic pathways [4]. The goal is to restore proper apoptotic signaling and effectively eliminate cancer cells [4]. In view of these considerations, it is possible that NO suppresses the proliferative potential of human melanoma cells by inducing apoptosis. This study investigated NO-induced apoptosis and the mechanisms through which this process is mediated in human melanoma A375 cells. This specific cell line was chosen because existing evidence indicates the potential involvement of NO in the development of melanoma and its relative insensitivity to induction of apoptosis by anti-cancer drugs [4].

EXPERIMENTAL

Cell cultures

The A375 melanoma cell line (a gift sample provided by Dr GN Wogan from the Massachusetts Institute of Technology in the USA), was cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. Cultures were maintained at 37 °C in a 5 % CO_2 atmosphere. All cell culture reagents were obtained from Lonza (Walkersville, MD).

Nitric oxide (NO) treatment

The A375 cells were plated at a density of 1 × 10^6 cells per 60 mm tissue culture plate to enable adhesion, 24 h prior to treatment and were exposed to 100 % NO through permeable silastic^{tm} tubing (0.058 in. i.d., 0.077 in. o.d., DOW Corning, Midland, MI) utilizing the NO delivery system. This delivery system controls NO dose and dose-rate using an NO flow regulator, at a steady state concentration of 7 μM [7]. A combination of 50 % oxygen and 5 % carbon dioxide was administered through a separate tubing loop to keep the oxygen level close to saturation. Total dose of NO delivered into the medium was regulated by adjusting exposure time, expressed as micromoles per minute (μM/min). Cells exposed to argon gas under identical conditions were used as negative controls.

Cell viability analysis

Cell viability was evaluated 24 h after treatment using trypan blue exclusion assay, and cell survival (S) was calculated using Eq 1.

S (%) = (CN/CA)100 …………………….. (1)

Where CN is the number of live cells in NO-treated group and CA is the number of live cells in argon control group.

Analysis of cell cycle

Cells were collected 24 h after NO treatment, washed twice with phosphate buffered saline (PBS), and fixed overnight in 70 % cold ethanol. Cells were subsequently resuspended in 1 % bovine serum albumin (BSA)-PBS solution containing 50 μg/mL propidium iodide (PI) and 50 μg/mL RNase. The cells were then incubated at 37 °C for 30 min before being analyzed using a Becton Dickinson FACScan. Cell fit analysis was used to determine the proportion of cells in a particular phase of the cell cycle.

Apoptosis analysis

Cells were harvested 24, 48 and 72 h after NO treatment by trypsinization and centrifugation, then analyzed in a Becton Dickinson FACScan (excitation at 488 nm) equipped with CellQuest software after staining with annexin V-FITC (Clontech Laboratories, Palo Alto, CA) and propidium iodide (Sigma Chemical, St. Louis, MO) [8]. Cells undergoing apoptosis were stained with annexin V, indicating early apoptosis, or with both annexin V and propidium iodide, indicating late apoptosis. Necrotic cells were stained only with propidium iodide, while living cells did not show any staining. Cells treated with argon gas served as negative controls, while those treated with 50 μM etoposide served as positive controls.

DNA fragmentation

Total DNA was isolated from the cells 24, 48 and 72 h after NO treatment, using a GenElute™ mammalian genomic DNA miniprep kit (Sigma Chemical, St. Louis, MO). Isolated DNA was suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 9.0) and quantified by absorbance at 260 nm. Fragmented DNA was loaded onto 1.8 % agarose gel containing Tris/Borate/EDTA (TBE) buffer, separated by electrophoresis for 2 h at 50 V, then photographed after staining with 0.5 ng/mL ethidium bromide.

Whole cell extract preparation and Western blot analysis

Cells were harvested 24 and 48 h after NO treatment and lysed in 450 µL of ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer containing Triss-buffered saline (TBS), 1 % Nonidet P-40, 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate (SDS), 0.004 % sodium azide, 1 mM phenylmethylsulfonyl fluoride (PMSF), and sodium orthovanadate, supplemented with 20 µg/mL protease inhibitor cocktail (Santa Cruz Biotechnology, Dallas, TX) for 30 - 60 min on ice, centrifuged at 10,000 g for 10 min at 4 °C. Protein concentration in the resulting supernatant was determined using the Bradford method (Bio-Rad Laboratories Inc., Hercules, CA) prior to conducting Western blot analysis. Approximately 60 µg of protein from whole-cell lysate or cytosolic fractions were denatured, separated by electrophoresis on 15 % sodium dodecyl polyacrylamide gel (SDS-PAGE), and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membranes were blocked with 5 – 7 % (w/v) nonfat dry milk in Tris-buffered saline/0.1 % Tween-20 (TBS/T, pH 7.6) for 1 - 3 h at room temperature. Subsequently, the membranes were incubated overnight at 4 °C with antibodies, appropriately diluted, against the following: p53 (1:1000, Oncogene, Cambridge, MA); Bax (1:1000, Cell Signaling Technology, Beverly, MA); DR4 (1:1000, StressGen Biotechnologies Corp, Victoria, BC, Canada); DR5 (1:1000, StressGen Biotechnologies Corp, Victoria, BC, Canada); Fas(CD95) (1:2000, StressGen Biotechnologies Corp, Victoria, BC, Canada); caspase 8 (1:1000, Cell Signaling Technology, Beverly, MA); caspase 9 (1:1000, BD PharMingen, San Diego, CA); caspase 3 (1:1000, Cell Signaling Technology, Beverly, MA), or actin (1:8000, Oncogene, Cambridge, MA). After two-10 mins washes with TBS/T, blots were incubated with the corresponding peroxidase-conjugated secondary goat anti-rabbit or mouse IgG (diluted 1:8000, Santa Cruz Biotechnology, Dallas, TX) for 1 h at room temperature, and washed two times for 5 mins and four times for 10 min. Enhanced chemiluminescence was determined by exposure to Hyperfilm ECL (GE Healthcare Bio-Sciences, Piscataway, NJ).

Statistical analysis

All experiments were repeated 2 to 4 times after optimizing the experimental conditions. The two-tailed Student's t-test was employed to statistically compare the responses between the NO-treated and argon control groups.

RESULTS

Loss of cell viability

Exposing A375 cells to a steady concentration of 7 µM of NO for 1.5, 3, 6, and 8 h corresponding to cumulative total NO doses of 630, 1260, 2520, and 3360 µM/min respectively, led to dose-dependent increases in cell lethality (Figure 1). Treatment with NO at doses below these cytotoxic levels did not result in any significant cell death and argon treatment had no effect on cell survival (Figure 1).

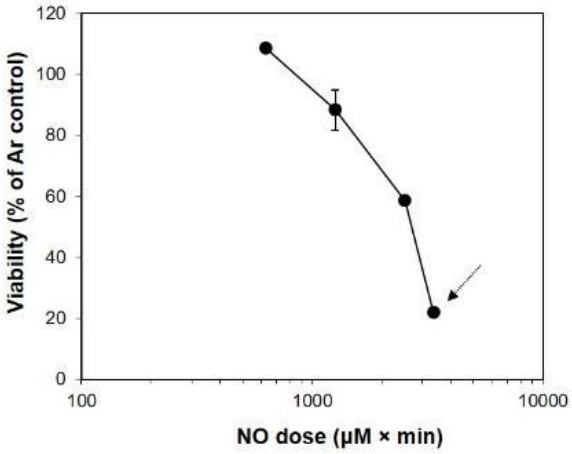

Figure 1: Dose-dependence of cell survival following exposure of A375 cells to cumulative dose (630-3360 µM/min) of NO. Survival was assessed using a trypan blue assay 24 h after treatment with NO. Cells treated with argon (Ar) served as negative controls. Data are presented in mean ± SD (n = 3)

Cell cycle changes

Exposure of A375 cells to 3360 µM/min NO caused arrest at the S phase ($p < 0.01$), while those in the G1 and G2/M phases decreased after treatment with NO. Cell cycle behavior of argon-exposed controls showed no significant

difference compared to unexposed cells (Table 1).

Apoptosis

Apoptosis was quantified using flow cytometry following annexin-V staining. Apoptotic response was significantly increased, peaking at 72 h after exposure to 3360 µM/min NO, with a maximum frequency of 73 % apoptosis, 8-fold higher compared to negative control group (Figure 2 A).

Internucleosomal DNA fragmentation

As fragmentation of genomic DNA is a significant biochemical characteristic of apoptosis, an analysis of genomic DNA was done after treating the cells. After treatment with 3360 µM/min, DNA ladder formation was evident with time intervals confirming that apoptosis played a significant role in cell death induced by NO (Figure 2 B).

Altered expression of proteins regulating intrinsic and extrinsic apoptotic pathways

Further investigation was conducted into the mechanisms that underlie cell death induced by NO, with emphasis on proteins that regulate the apoptotic process. Expression of p53 and Bax proteins was initially examined (Figure 3 A). Cells treatment with 3360 µM/min NO led to a significant increase in p53 expression progressively for 48 h. The results also showed a slight increase in Bax protein (a proapoptotic member of the Bcl-2 family) which is a significant target for p53 (Figure 3 A). Furthermore, activation of DR4, DR5, Fas (CD95), and caspase 8 was investigated to assess the involvement of extrinsic apoptotic pathway in treatment-induced cell death. The Western blot revealed an increase in DR4 and Fas (CD95) proteins, as well as a decrease in procaspase 8 proteins after treatment (Figure 3 B). The maximum and minimum levels of these proteins after 48 h period following treatment were comparable to those observed for the intrinsic pathway protein. These findings indicate that both intrinsic and extrinsic apoptotic pathways play a role in cell death induced by NO treatment.

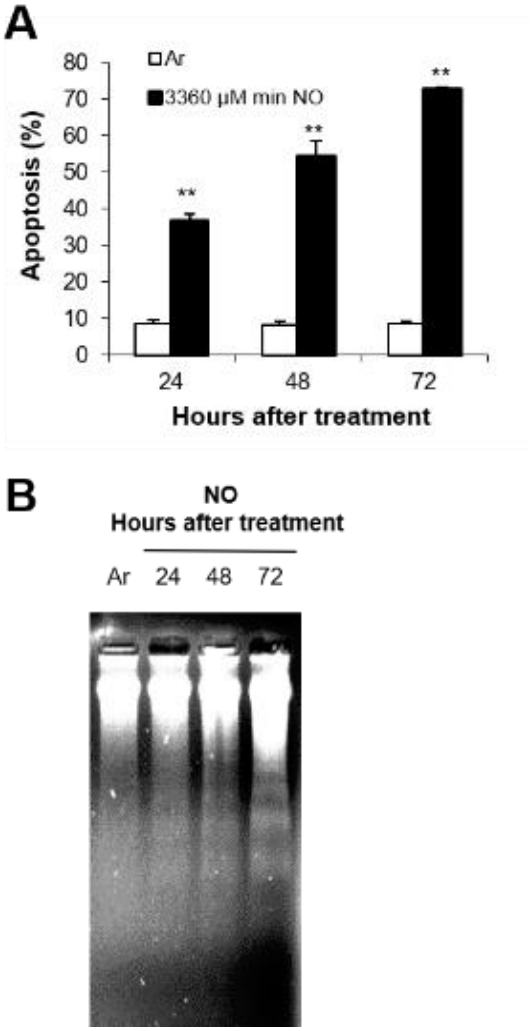

Figure 2: Induction of apoptosis by 3360 µM/min NO (arrow in Figure 1). Apoptosis was determined by annexin V versus PI staining (A) and internucleosomal DNA fragmentation (B) of A375 cells after treatment with 3360 µM/min NO. Results are presented as mean ± S.D (n=3). *P < 0.05 and **p < 0.01 vs. negative control group

Table 1: Effect of NO on cell cycle in A375 cells 24 h after treatment

Total NO dose	Distribution (mean ± SD, %)		
(µM/min)	G_1/G_0	S	G_2/M
Unexposed	37.2±0.8	34.3±10.9	30.6±9.3
Argon	36.0±3.5	32.8±15.1	31.2±15.8
1260	35.0±7.5	35.1±9.9	30.0±10.4
2520	32.9±6.1	47.4±6.6	19.8±6.6
3360	6.7±2.1	75.3±10.1**	17.9±8.2

Results are displayed as a percentage of control cells (mean ± SD, n=3). **p < 0.01, compared to negative control group

Activation of caspase-dependent cascade (caspase 9 and caspase 3)

Nitric oxide treatment resulted in degradation of the precursor form of both caspase 9 and caspase 3 over a period of 24 - 48 h (Figure 3 C).

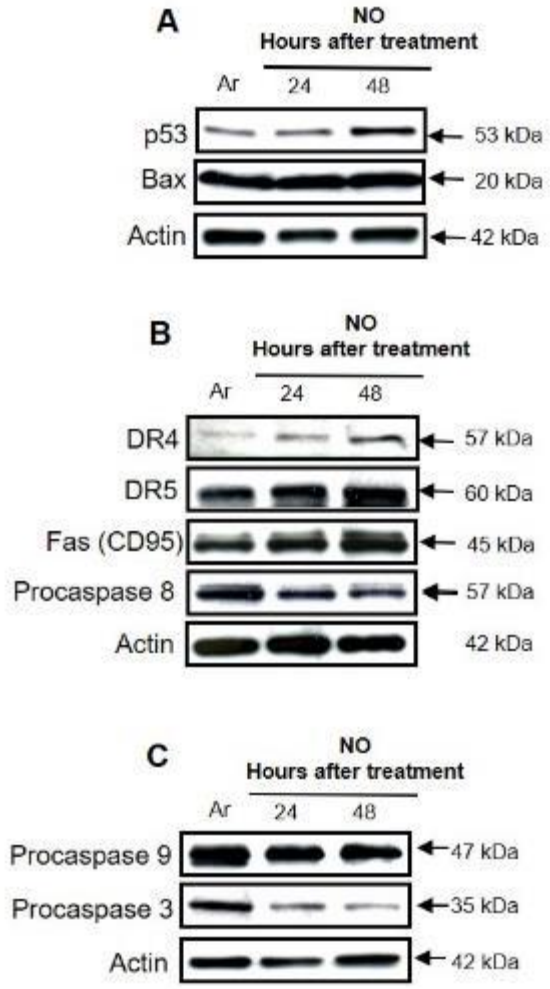

Figure 3: Representative Western blot showing changes in levels of p53 and Bax (A); DR4, DR5, Fas (CD95) and Procaspase 8 (B); and participation of caspases 9 and 3 on apoptosis (C) after treatment with argon (Ar) and 3360 µM/min NO (arrow in Figure 1). Results represent findings from two independent experiments

DISCUSSION

At low concentrations, NO serves as a signaling molecule involved in many physiological processes, whereas excessive NO causes damage to cellular DNA, proteins and lipids, which in turn trigger downstream signaling pathways eliciting repair or cell death through necrosis or apoptosis [4,9]. Previous studies have shown that NO induces proliferation of solid human tumors and inhibition of apoptosis [9]. One example is the inhibition of melanoma cell growth observed upon scavenging endogenous NO, which was restored by using a NO donor [10]. However, NO-induced melanoma apoptosis and its mechanism is not clearly understood. This study investigated the mechanisms associated with NO-induced apoptosis in A375 human melanoma cells. This study revealed that NO production and expression of NOS proteins, particularly iNOS, in A375 cells confirmed a potential association between iNOS and NO levels with melanoma cell proliferation and survival which is in tandem with a previous study [11]. Additionally, it was found that inhibitors and scavengers of NO inhibit A375 cell proliferation, suggesting that endogenous NO might play a significant role in this process [11]. However, in apparent contrast to these results, exogenous NO inhibits cell growth and promote apoptosis in cell line A375, indicating that NO have dual effects in melanoma, including pro- and anti-tumor activity. For exogenous NO treatment, the controlled delivery of NO under conditions designed to mimic *in vivo* situation found in inflamed tissue was used [12].

In earlier related investigations, it was discovered that both concentration and cumulative total dose were critical in initiating NO toxicity [13,14]. This study demonstrated that at a steady-state concentration of 7 µM, thresholds for NO-induced cell death were approximately 1260 µM/min in A375 cells. If the thresholds were not exceeded, cells proliferated normally. As the doses were increased above the threshold values, NO induced dose-dependent cellular toxicity and death. Dual effects of NO are linked to its concentration, and this mechanism of NO in tumor biology may explain why endogenous NO plays a crucial role in A375 cell proliferation, while exogenous NO diminishes cell survival.

Apoptosis contributed to only a small percentage of the reduced cell survival in this cell line. For example, treating A375 cells with 3360 µM/min of NO led to approximately 22 % cell survival compared to argon-treated control cells; however, apoptosis 24 h after treatment accounted for only 33 % of the cell loss observed. The remaining cell loss was attributed to NO-induced cell cycle arrest at the S phase. Cell cycle arrest induced by DNA damaging agents is assumed to give the cell time to repair potentially mutagenic and cytotoxic DNA damage [15,16]. The S phase arrest is believed to occur due to damage in DNA replication, which suppresses DNA synthesis or elongation [17,18]. If the cell can repair the damage, replication resumes, leading to the production of replacement cells. However, if the damage is

irreparable, apoptosis may be initiated to eliminate irreversibly injured cells [17,18].

Mammalian cells have two major apoptotic pathways which include the intrinsic and extrinsic pathways. Both pathways converge at the activation of the initiator and effector caspases. Expression of p53 increases cell sensitivity to apoptosis and regulates Bcl-2 family proteins and the release of mitochondrial cytochrome c, which modulate the intrinsic pathway of apoptosis [4]. Association of cytochrome c with an adapter molecule, Apaf1, triggers the activation of caspase 9 in the cytoplasm, which then activates downstream caspases [4]. In the extrinsic pathway, specific death ligands bind to their corresponding cell surface receptors, such as Fas (CD95), tumor necrosis factor receptor (TNFR), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors DR4 and DR5. This activates downstream pathways through recruitment of initiator caspase 8, which subsequently cleaves further effector caspases [4]. Absence of the active form (43 and 18 kD) of caspase 8 in the Western blot analysis may be attributed to increased protein degradation.

To comprehend the p53-related mechanisms through which NO activates the apoptotic pathway, expression of p53 and Bcl-2 family was investigated. Exposure to NO stimulated both p53 and Bax expression in A375 cells. The activation of caspase 9 was also observed together with caspase 3, suggesting that NO induces apoptosis through the mitochondrial pathway. Upregulation of DR4, DR5, and Fas (CD95), as well as activation of caspase 8, suggest that NO activates both mitochondrial and death receptor pathways, working simultaneously to induce apoptosis.

CONCLUSION

The results show that apoptotic responses are induced by exogenous NO production in A375 cells and involves both extrinsic and intrinsic pathways. While these findings generally support the potential application of NO as cancer chemopreventive or therapeutic agent, this model provides a framework for future studies to specifically define the mechanisms through which it acts.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. Seo Hyun Moon performed the experiments. Min Young Kim designed the experiment. All authors co-prepared the manuscript.

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