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Nitric oxide inhibits gastric cancer cell growth through the modulation of the Akt pathway

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Abstract. Nitric oxide (NO) is involved in a number of physiological and pathological processes. As an important biological mediator, NO has been the focus of cancer study for its function in tumorigenesis, tumor progression and death. The effects of NO on tumor cells are multifaceted, but many details underlying these effects are not yet well understood. In this study, we demonstrate that NO directly suppresses the growth of BGC-823 cells by inducing G0/G1 phase arrest in a dose- and time-dependent manner. We also reveal that G0/G1 arrest results from the NO-induced disruption of the cell cycle balance, which is mediated by the up-regulation of $p21^{waf1/cip1}$ and the down-regulation of cyclin D1 and E and PCNA. Exposure of BGC-823 cells to various sodium nitroprusside (SNP) concentrations for 24 h or to 1 mmol/l SNP for various times resulted in a marked decrease in the level of phospho-Akt and increase in the level of phospho-ERK. In brief, the NO-induced cell growth suppression and G0/G1 arrest are mediated through the regulation of cell cycle-related proteins, which may depend on the inactivation of Akt signaling. This may be one mechanism through which NO inhibits gastric cancer cell growth.

Introduction

Nitric oxide (NO), a highly versatile free radical gas of physiological significance, is capable of controlling multiple cellular signaling processes in organisms ranging from bacteria to humans (1-3). It has been reported that NO is capable of inhibiting cell proliferation in certain cell types and conditions, but stimulates cell proliferation in others (4,5). The dual role of NO depends on its concentration, action time and effector sites of generated NO (6). However, the cellular targets for NO and the related molecular mechanism remain unclear. Thus, it is essential to understand the manner in which NO controls cell proliferation.

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Abnormal regulations of cell cycle and cell proliferation are important mechanisms of tumorigenesis. Cell cycle progression is mediated by cyclin-dependent kinases (CDK) (7). Progression in G1 phase and entry into S phase are related to the activity of CDK2/cyclin E complex (8). Cyclin E expression increases during G1 and peaks at G1-to-S transition. Cyclin D1 and its partners CDK4 and CDK6 have been found to be most closely linked to the late G0/G1 phase checkpoint (9,10). Whether cells transit from G1 to S phase depends largely on the accumulation of cyclin D1 in the G1 phase. The CDK activity is also affected by cyclin-dependent kinase inhibitors (CKIs) (11). $P21^{waf1/cip1}$, a CKI that interferes with cyclin E activity, is present in CDK complexes of proliferating cells during all phases of the cycle. The conversion of active into inactive complexes is achieved through alteration of the ratio of $p21^{waf1/cip1}$ to CDK (12). The MAPK/ERK pathway has classically been associated with anti-proliferative signaling. Transient or cyclical ERK activation has been linked to cell cycle progression, whereas sustained levels of ERK activity can lead to cell growth arrest and differentiation (13). ERK phosphorylation and the up-regulation of $p21^{waf1/cip1}$ are known to be central in NO-mediated smooth muscle cell anti-proliferation (14). It has also been demonstrated that the NO-induced deactivation of the Akt pathway plays a central role in cell growth and survival (15). NO is known to mediate the ERK pathway and inhibit Akt signaling, but the question whether it modulates gastric cancer cell growth via these pathways has yet to be investigated. In this study, BGC-823 cell lines were selected to investigate the effect of NO, which may aid in the understanding of the effect of NO on this type of malignant tumor cell and the related mechanisms.

Materials and methods

Cell lines and reagents. The human gastric cancer cell line, BGC-823, was provided by the Institute of Cell Biology (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) was from Gibco (Grand Island, NY, USA) and new-born calf serum (NBCS) was from Minhai Bio-engineering Co. (Lanzhou, China).

Antibodies against PCNA, cyclin D1 and E, $p21^{waf1/cip1}$, p-Akt and Akt were from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). Antibodies against proliferating cell nuclear antigen (PCNA), p-ERK and ERK were from Boster Biological Technology Ltd. (Wuhan, China). Peroxidase-conjugated

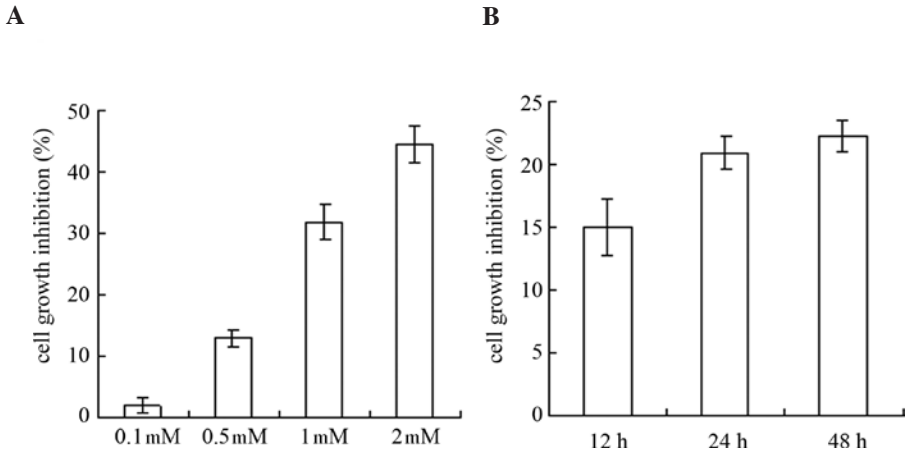


Figure 1. NO inhibits the proliferation of BGC-823 cells in a dose-dependent manner. Cells were treated with (A) various SNP concentrations for 24 h or (B) 1 mM SNP for various times. Cell proliferation was examined using the MTT assay. Values are the means ± SE of three different experiments.

secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Antibody against β-actin (glyceraldehyde phosphate dehydrogenase) was from Kangcheng (Hangzhou, China). Electrochemiluminescence (ECL) reagents were from Amersham Biosciences (Buckinghamshire, UK). All other reagents used were of analytical grade.

Cell culture. BGC-823 cells were cultured in DMEM supplemented with 10% NBCS and maintained at 37˚C in a humidified incubator with 95% air and 5% CO_2. The medium was changed every two days, and the cells were subcultured to confluence. BGC-823 cells were exposed to 0.1, 0.5, 1, 2 mM sodium nitroprusside (SNP) for 24 h, or 1 mM SNP for 12, 24 or 48 h.

Western blotting. The cells were harvested and homogenized with an ultrasonic homogenizer in sample buffer. The homogenates were heated in boiling water for 5 min, separated by SDS-PAGE gels and blotted onto a polyvinyl difluoride (PVDF) membrane. The PVDF membrane was blocked with 3% bovine serum albumin in TBS-T for 1 h at room temperature (RT). Incubation with the primary antibody was conducted overnight at 4˚C, and incubation with the secondary antibody was for 1 h at RT, with three washes following each incubation. ECL reagents were used to reveal the positive bands on the membrane. The bands were detected by Typhoon 9400 (GE Healthcare, USA).

MTT assay. A total of $1x10^4$ cells (in 100 μl complete DMEM) were seeded in one well of a 96-well plate. Following attachment, the cells were washed and serum-starved overnight. Thereafter, the cells were incubated in 0.1, 0.5, 1 or 2 mM SNP for 24 h or 1 mM SNP for 12, 24 or 48 h. Approximately 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyl tetrazolium bromide (MTT) (5 mg/ml) were added to each well, and the plate was incubated for 4 h. Dimethylsulfoxide (DMSO) (150 μl) was added to the wells to dissolve the formazan crystals, and the optical density at 490 nm was measured. Data were presented as a percentage of control proliferation, and a value of $P<0.05$ was considered significant.

Flow cytometric analysis of the cell cycle. BGC-823 cells were washed twice with PBS and suspended in 4 ml of 75% ethanol. Prior to analysis, the cells were washed twice with PBS and incubated with RNase A (10 μg/ml) at 37˚C for 30 min. After being stained with Pi-staining solution in the dark for 30 min at RT, the cells were analyzed by flow cytometry on a FACScalibur (Becton-Dickinson).

Statistical analysis. Data were expressed as the means ± SE. Statistical significance was examined with the Student's t-test. A value of $P<0.05$ was considered significant. All other results are representative experiments that were reproduced at least three times.

Results

NO dose-dependently inhibits the cell proliferation of gastric cancer cells. To assess whether NO regulates growth inhibition, we incubated cells with SNP. The cytotoxic effect of NO on the growth of gastric cancer cells was determined by MTT assay. The IC50 value in BGC-823 cells was 1.32 mM after 24 h of SNP treatment (data not shown). At the same time it was shown that NO has an inhibitive effect on the proliferation of cells at various concentrations of SNP for 24 h in a dose-dependent manner (Fig. 1A). At a concentration of 1 mM, SNP exhibited a tendency to inhibit the BGC-823 cells at 24 and 48 h of treatment (Fig. 1B). However, our results showed that there was no significant difference between the two time-points ($P<0.05$). These results suggest that NO inhibits cell proliferation in a dose-dependent manner.

NO alters the cell cycle of gastric cancer cells. In order to investigate the reason why the proliferation of BGC-823 cells was inhibited, the cell cycle of BGC-823 cells treated with various concentrations of SNP for 24 h or with 1 mM SNP for various times, was analyzed. SNP decreased the number of cells in the S phase and increased the number of cells in the G0/G1 phase, an effect that occurred in a dose-dependent manner (Table IA). The BGC-823 cells underwent cell cycle arrest as early as 12 h after 1 mM SNP treatment, while SNP

Table I. NO induces cell cycle arrest in a dose-dependent manner in BGC-823 cells.

A, SNP concentration

Concentration (mM)	Phases of the cell cycle (%)		
	G0/G1	S	G2/M
0	52.10±0.32	46.77±0.34	1.13±0.43
0.1	56.32±1.35	42.75±0.75	0.93±0.37
0.5	62.65±0.69*	36.10±0.89	1.25±0.19
1	67.78±1.58*	31.23±1.01*	0.99±0.51
2	75.05±2.07*	23.86±2.51*	1.09±1.04

B, SNP exposure time

Time (h)	Phases of the cell cycle (%)		
	G0/G1	S	G2/M
0	50.88±1.36	47.01±1.21	2.09±0.03
12	56.11±1.15	42.43±1.03	1.36±0.14
24	62.99±1.57*	35.52±1.37*	1.42±0.18
48	64.07±0.59*	34.80±0.89*	0.97±0.01

Cells were treated with (A) various SNP concentrations for 24 h or (B) 1 mM SNP for various times. Values are the means ± SE of three different experiments. *P<0.05.

hardly changed the number of cells in each phase at 48 or 24 h of treatment (Table IB) (P<0.05). These results clearly demonstrate that NO delays the conversion from the G0/G1 to the S phase.

NO suppresses cell-cycle protein expression. To further study the effect of NO on proliferation, we examined the expression of proteins involved in the cell cycle at the G1-S transition. Treatment with SNP resulted in a marked enhancement in the expression of $p21^{waf1/cip1}$ in a time- and dose-dependent manner (Fig. 2). Analysis of the expression of cyclin E and D1 indicated that NO treatment of BGC-823 cells resulted in time- and dose-dependent reduction of their expression (Fig. 2). When we examined the expression of PCNA, a protein required for replication, that is the marker of proliferating cells, we observed a comparable result. The PCNA levels were reduced at 12 h following SNP treatment (Fig. 2B). The reduction of the expression of cyclin E, cyclin D1 and PCNA indicates that NO suppresses cell proliferation by blocking G1-S phase transition in the cell cycle. Consistent with the initial inhibition of cell growth, SNP treatment resulted in G0/G1 arrest, which manifested as an increased G0/G1 content and decreased S phase content in a dose- and time-dependent manner. Thus, our data demonstrate that NO induces cell cycle arrest at the G0/G1 phase.

NO inhibits Akt and activates ERK. The mechanism under NO-induced cell growth inhibition and cell cycle arrest in BGC-823 cells was investigated. The MAP kinase cascade is important for cell proliferation, differentiation and apoptosis (16). The Ser/Thr protein kinase, Akt, is recognized as one of the main downstream effectors of PI3K that promote cell proliferation and survival (17-20). In order to verify the effect of SNP on the activities of ERK and Akt, we performed experiments measuring the expressions and the phosphorylation levels of ERK and Akt in gastric cancer cells. As shown in Fig. 3, exposure of BGC-823 cells to 1 mM SNP for 12-48 h resulted in a marked decrease in Akt phosphorylation. Simultaneously, a decreased phospho-Akt level was observed with the increase of the concentration of SNP. However, NO-induced time- and dose-dependent increases in phospho-ERK were observed. These results indicate that the effect of NO on BGC-823 cell proliferation does not involve the ERK pathway and that NO induces cell cycle arrest through the inhibition of phospho-Akt levels.

Discussion

It has been demonstrated that NO is involved in diverse processes in cellular signaling and in the potentiation of numerous tumors. Studies have already revealed that NO inhibits cell growth and induces apoptosis in various cells, including tumor cells. Similar results were obtained in our study by evaluating the effect of SNP, a NO donor, on the

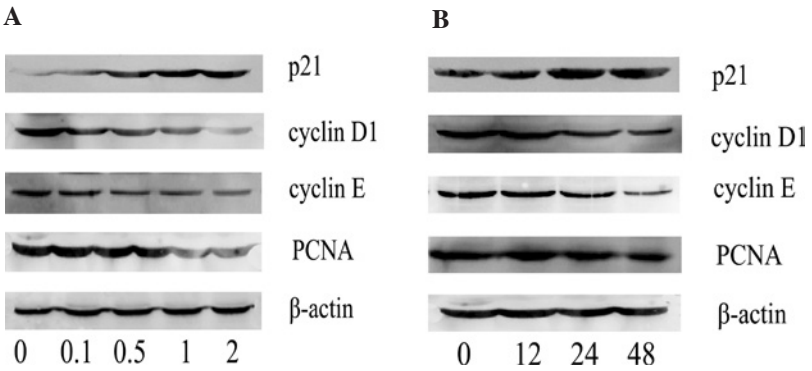


Figure 2. Effects of NO on G1/G1 cell cycle regulatory proteins in BGC-823 cells. Cells were treated with (A) various SNP concentrations for 24 h or (B) 1 mM SNP for various times and the expression of $p21^{waf1/cip1}$, cyclin D1, and cyclin E was determined by Western blot analysis. The experiments were repeated at least three times with similar results.

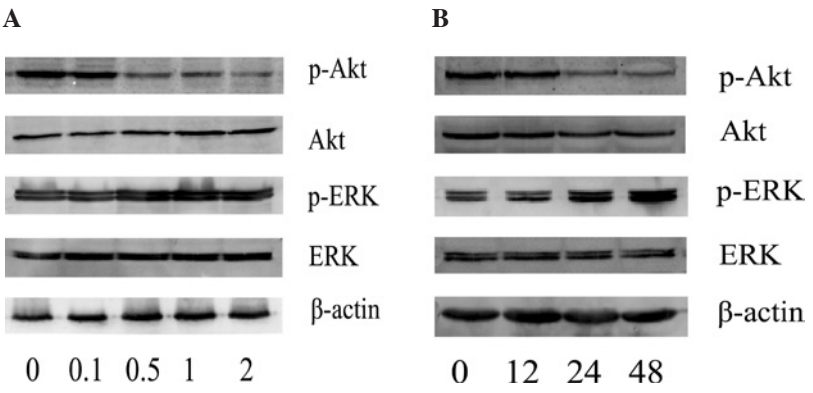


Figure 3. Effects of NO on the activation of ERK and Akt in BGC-823 cancer cells. Cells were treated with (A) various SNP concentrations for 24 h or (B) 1 mM SNP for various times and the expression of the phospho-protein was determined by Western blot analysis. The experiments were repeated at least three times with similar results.

proliferation in BGC-823 cells. BGC-823 cells were treated with various concentrations of SNP for various times. As shown in Fig. 1, the proliferation of BGC-823 cells was significantly inhibited by NO, with more potent blocking when increasing concentrations of SNP were used. Furthermore, our results have shown that the significant growth-inhibiting effect of NO on BGC-823 cells was observed when the cells were exposed to SNP for as long as 48 h, indicating that NO-mediated inhibition of cell proliferation lasts for a prolonged period of time.

The cell cycle analysis revealed that, in SNP-treated BGC-823 cells, the significant decrease of S phase cells was accompanied by an increase of G0/G1 phase cells and no change of G2/M phase cells. Thus, the decrease of cell numbers in the S phase was clearly due to the increase of cells in the G0/G1 phase, and the blockade effect of NO occurred at the G1-S transition. The data indicated that there was no significant difference between the cell numbers in each phase from 24 to 48 h at the same concentration.

The changes of multiple regulators associated with the cell cycle were investigated in order to further elucidate the mechanism of action of NO. PCNA has often been considered as a marker of cells undergoing the proliferation process (21). Cyclin E and D1 are required for cell cycle G1/S transition (22). The expression of cyclin D1 is modulated by $p21^{waf1/cip1}$. As shown in Fig. 3, the levels of cyclin D1 were suppressed as those of $p21^{waf1/cip1}$ increased, which was in agreement with the known regulatory relationship between the two proteins. Our data also revealed that NO down-regulated the levels of cyclin E in a time- and dose-dependent manner, thus providing a possible explanation for the observed S-phase arrest induced by NO. Simultaneously, 12-48 h treatment of SNP significantly down-regulated the expression of PCNA, which controls the progression of cells through the S phase of the cell cycle. Taken together, NO induced cell growth arrest via down-regulation of the expression of cyclin D1 and E and PCNA, and up-regulation of $p21^{waf1/cip1}$ expression in gastric cancer cells.

The blockade of the MAP kinase cascade is known to inhibit cell proliferation in certain cell populations (23-25). However, the effect of NO on gastric cancer cell proliferation does not seem to be involved in the inhibition of this pathway, as NO increases ERK activation in BGC-823 cells. One possibility is that the NO-mediated activation of the MAP kinase cascade is involved in other physiological events, such as survival and differentiation (26-27). Cyclin E and D1 and $p21^{waf1/cip1}$, the key signaling downstream molecules, were activated in response to MAPK activation, ultimately leading to cell cycle deceleration and the activation of cell anti-proliferation. Our results indicate that Akt may be involved in NO-induced anti-proliferation, as the levels of phospho-Akt were significantly reduced by NO following the exposure of BGC-823 cells to various SNP concentrations for various times.

In conclusion, we observed that NO inhibited the growth of cancer cells by blocking the cell cycle at the G0/G1 phase. We also provide mechanistic evidence that the NO-induced G0/G1 arrest is mediated through regulation of the cell cycle-related protein, which is likely to be associated with inactivation of the Akt pathway. However, the association between the down-regulation of cell cycle regulatory molecules and the phosphorylation changes of ERK and Akt in NO-treated cells is not fully understood and warrants further investigation.

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