

Inhibition of Apoptosis by Nitric Oxide in MCF-7 Cells

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Nitric oxide (NO) is a diffusible, multifunctional and transcellular messenger that has been implicated in numerous physiological and pathological conditions. It has been reported that NO induced apoptosis in tumor cells, macrophage cells and inhibited apoptosis in normal cells, endothelial cells. To examine whether NO could induce apoptosis in MCF-7 cells, cells were treated with SIN-1 (3-morpholinosydnonimine), NO donor. Cell viability did not change in SIN-1 treated cells for 48 h and there was no significantly changes in cell cycle progression or growth pattern by FACS analysis. But p53 protein, an apoptosis-related factor, increased SIN-1 treatment time dependently. Bcl-2, MDM2 and p21 were also accumulated. Bax level did not change. A major role of inhibiting apoptosis by NO in MCF-7 cells, cobalt chloride (CoCl₂) was added to cells preincubated with SIN-1. Whereas CoCl₂ treated cells underwent apoptosis, for 24 h SIN-1 preincubated cells were not induced apoptosis. Inactivated proteins, MDM2 and bcl-2, by CoCl₂ levels also increased in SIN-1 pre-treated cells. These results suggested that SIN-1 blocked p53 by MDM2 activation and inhibited apoptosis by inducing p21 and bcl-2 expression.

Key words : MCF-7, Nitric oxide, p53, MDM2, bcl-2

Introduction

Cell numbers are regulated by a balance between proliferation, growth arrest and programmed cell death (apoptosis) [7,23,32]. Apoptosis is a distinctive form of cell death designed to eliminate unwanted cells through the activation of a coordinated and highly regulated process. Recently, nitric oxide (NO) has received a great deal of attention as an important regulator of apoptosis.

Nitric oxide, a potentially toxic molecule, has been implicated in a wide range of diverse physiologic and pathologic processes. It is catalytically produced by different NO synthase (NOS) isoforms such as endothelial NOS, neuronal NOS and inducible NOS [18]. Because of the ubiquitous distribution of the NO synthases and diverse chemical reactivity of NO in biological systems, understanding of the definite role of NO and its signaling mechanism have been emphasized. Numerous investigators have reported that NO can promote apoptosis through the induction of damages to DNA in some cells, whereas it inhibits apoptosis in other cells. NO stimulates the expression of enzymes and transcription factors involved in DNA repair and modulation

of apoptosis such as the tumor suppressor p53 [10,18].

Several studies have suggested that p53 protein suppress cancer cell growth in vitro and may induce apoptosis when accumulated [22,30,33]. Also, p53 induced apoptosis in MCF-7 cells by oxidative stress, H₂O₂ [1,26]. This study was performed to examine whether NO can promote apoptosis in breast cancer cells or not. The anti-apoptotic effect and role of NO were investigated in breast cancer cells, using by cobalt chloride as an apoptosis inducer [11,12,14,29].

Materials and Methods

Cell lines, culture, and treatment

Human MCF-7 breast cancer cell line was obtained from American Type Culture Collection (ATCC, USA). They were maintained as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/ streptomycin (Gibco BRL Life Technology, USA) at 37°C under a humidified atmosphere containing 5% CO₂. Cells were treated with 1 mM 3-morpholinosydnonimine (SIN-1, Sigma, USA) in PBS for each hour such as 30 min, 3 hr, 24 hr, or 48 hr. Otherwise, cells were incubated with 800 μM COCl₂ for 48 hr, 47½ hr, 42 hr, 36 hr, or 24 hr after 1 mM SIN-1 treatment for 30 min, 3 hr, 6 hr, 12 hr, or 24 hr, respectively.

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Cell viability assay

MCF-7 cells were incubated in 96-well for 24 h and treated with SIN-1 and CoCl_2 . After incubating for 24 hr, the cells were treated with growth medium (DMEM with 10% FBS and 1% antibiotics) and 1 mg/ml of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma, USA). Cells were incubated at 37°C and 5% CO_2 for 4 hr. The medium was aspirated and the formazan crystals, which are formed from MTT by NADH-generating dehydrogenases in metabolically active cells, were dissolved in 200 μl DMSO. An Microplate Reader (Bio-rad, USA) determined absorbance at 570 nm, with a reference of 750 nm. Viability was defined in relation to that of the quiescent cells prior to treatments with the equation. The assay was performed in triplicate. Data were analyzed with a two-tailed t test, with statistical significance defined as $P < 0.05$.

FACS analysis of apoptosis

Cells pretreated with SIN-1 for 4 h, or untreated, were treated with CoCl_2 . Both attached and unattached cells were harvested with trypsin/EDTA, washed with cold PBS containing 1% bovine serum albumin (BSA, Sigma, USA). The cells were fixed by the addition of cold 70% ethanol for over 4 hr at 4°C. Prior to analysis, the cells were washed and resuspended in 1% BSA. After washing, the cells were stained with propidium iodide (50 $\mu\text{g}/\text{ml}$) in the presence of RNase (100 $\mu\text{g}/\text{ml}$) for 40 min. The stained cells were analyzed at 488nm on an EPICS-XL cytometer (Beckman Coulter) equipped with an air-cooled 20 mW argon laser. Cell cycle was analyzed using Multicycle software (Advanced version, Phoenix Flow Systems, USA). The fraction of cells in the hypodiploid or sub G_0 - G_1 peak was considered apoptosis.

Western blotting

For protein analysis, cells plated in 10 cm-diameter dishes were harvested and washed with the phosphate-buffered saline (PBS; 2.6 mM KH_2PO_4 , 4.1 mM NaH_2PO_4 , 135 mM NaCl, pH 7.4) and then scraped in lysis buffer (250 mM NaCl, 50 mM Hepes (pH 7.0), 0.1% Nonidet P40, 1% PMSF, 1 mg/ml Aprotinin, 1 mg/ml Leupeptide) for 30 min on ice. Lysates were clarified by centrifugation at 12,000 rpm for 10 min at 4°C and the supernatant was obtained. The protein contents of the lysate were determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, USA). Thirty μg protein was mixed with an equal volume of electro-

phoresis buffer (100 mM Tris-HCl, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol). After heating, the protein was resolved on polyacrylamide SDS gels and transferred to nitrocellulose membrane (Amersham Pharmacia Biotech Inc, USA). The membranes were blocked for 1 hr at room temperature with blocking reagent (5% non-fat milk, 0.05% Tween 20 in TNE buffer, pH 7.5). The membranes were incubated with primary antibody. After three washes, the membranes were incubated for 1 hr with the corresponding secondary antibody, diluted in the above blocking reagent. After three final washes, the membranes were treated with chemiluminescence reagent (ECL, Amersham Pharmacia Biotech Inc, USA). All the procedures were done at room temperature. Antibodies against p53, bcl-2, MDM2, bax and p21 were purchased from Oncogene Research Products (Germany) and Santa-cruz Biotechnology, Inc (USA).

Results

Effects of chemicals on cell viability

SIN-1 did not induce cell death in MCF-7 cells, in spite of sufficient incubation time, as determined by MTT assay. Even after 48 hr of incubation with 1 mM SIN-1, cell viability was not reduced at all (Fig. 1A). Cell death was detected in the cells treated with 800 μM CoCl_2 for more than 24 hr. After incubation for 24 hr incubation with 800 μM CoCl_2 , cell viability began to reduce to 70% of control, and after 48 hr incubation it reduced further to 40%. As shown in Fig. 1C, SIN-1 and CoCl_2 treatment reduced cell death significantly and cell viability kept on increasing to more than 70% of control after 24 hr.

SIN-1 does not induce apoptosis

To establish a relationship between the change in the various molecules and the cell cycle or apoptotic pathways, the proportion of cells entering different phases of the cell cycle and the amount of apoptosis in the treated cell population were studied by flow cytometric analysis. The results showed that SIN-1 did not affect cell cycle. The percentage of MCF-7 cells in G_1 , S, and G_2 phase were similar to control cells and the sub G_0 - G_1 peak representing apoptotic cells was undetectable (Fig. 2, Table. 1), but in 48 hr SIN-1 treatment cells, an apoptotic peak presented 1.4%. SIN-1 has been shown to cause accumulation of the nuclear p53 in MCF-7 cells after 30 min. Level of p53 increased time-dependently.



Fig. 1. Effects of SIN-1 and CoCl₂ on MCF-7 cells growth. A, B: Cells were treated with 1 mM SIN-1 and 800 μM CoCl₂ for 30 min, 3 hr, 24 hr, 48 hr. C: Cell viability in the cells preincubated with 1 mM SIN-1 for 30 min, 3 hr, 24 hr and then treated 800 μM CoCl₂ for 47½ hr, 45 hr, 24 hr. **p*<0.05 vs. untreated control, #*p*<0.05 vs. the CoCl₂ treated group.

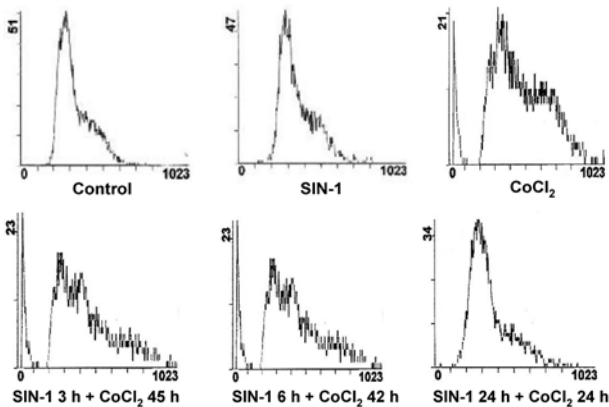


Fig. 2. FACS analysis shows that SIN-1 inhibits apoptosis on MCF-7 cells. Cells were treated with 1 mM SIN-1 and 800 μM CoCl₂ for 24 hr. To detect if SIN-1 inhibits apoptosis in MCF-7 cells, cells were pretreated with 1 mM SIN-1 for 30 min, 3 hr, 24 hr and then treated 800 μM CoCl₂ for 47½ hr, 45 hr, 24 hr.

Table 1. Percentage of cells in G₁, S, G₂, and apoptosis (Sub G₀-G₁)

Treatment	Cells in G ₁ , S, G ₂ , and apoptosis (%)			
	G ₁	S	G ₂	Apoptosis
Control	45.0	45.0	10.1	0.8
SIN-1 24 hr	41.3	53.5	5.2	0.8
SIN-1 48 hr	42.5	50.2	7.3	1.4
CoCl ₂ 48 hr	34.9	33.3	31.8	15
SIN-1 3 hr + CoCl ₂ 45 hr	23.3	53.1	23.5	12.5
SIN-1 6 hr + CoCl ₂ 42 hr	38.4	37.2	24.4	7.7
SIN-1 24 hr + CoCl ₂ 24 hr	55.8	12.6	31.6	0.5

Changes in the levels of proteins such as p21, MDM2 and bax regulated by p53 were studied in SIN-1 pretreated MCF-7 (Fig. 3). In cells, the p21 and MDM2 protein levels were found to increase (Fig. 3A). As shown Fig. 3B, the anti-apoptotic gene bcl-2 was also detected. Although level of

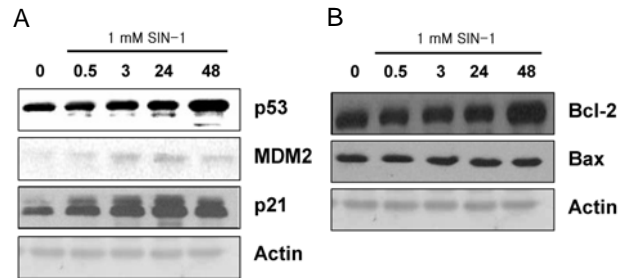


Fig. 3. Expression of p53, MDM2, p21, bcl-2 and bax in 1 mM SIN-1 treated cells. Untreated cells, a; 30 min. b; 3 hr, c; 24 hr, d; 48 hr, e Actin was used as loading control.

bcl-2 was accumulated, bax was found to remain unchanged compared to the control.

SIN-1 inhibits apoptosis

The previous results showed that SIN-1 did not induce apoptosis in MCF-7 cells. To confirm that SIN-1 protects apoptosis in MCF-7 cells, cells were treated CoCl₂. Already, some studies have shown that CoCl₂ induce apoptosis [11,12,14,29]. Apoptosis also examined by FACS analysis and western blotting in CoCl₂ treated cells. In the 48 hr treatment with CoCl₂, cell cycle was changed. The sub G₀/G₁ peak was 15% (Fig. 2, Table 1). The levels of p53 and bcl-2 were shown as an apoptotic pattern in CoCl₂ treated cells. p53 increased and bcl-2 decreased in a time dependent manner (Fig. 4A). Then MCF-7 cells were preincubated with SIN-1 and then treated with CoCl₂. SIN-1 was pretreated for 30 min, 3 hr, 6 hr, 12 hr, or 24 hr and CoCl₂ was treated for 47½ hr, 45 hr, 42 hr, 36 hr, or 24 hr. As shown Table. 1, the sub-G₁ peak was found to decrease, as cells were preincubated with SIN-1 for 24 hr and CoCl₂ treatment for 24 hr. The sub G₀/G₁ peak reduced to 12% and 7.7% in 3 hr and 6 hr SIN-1 treatment and further reduced to 0.5% in 24 hr SIN-1 treatment

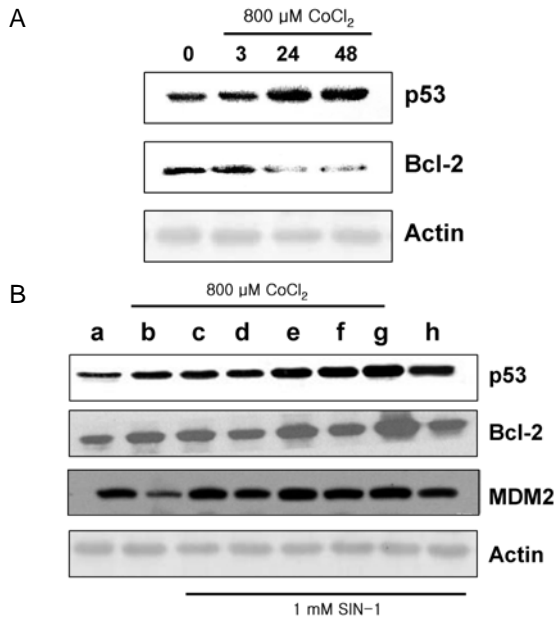


Fig. 4. A: Change of p53 and bcl-2 levels in 800 μ M CoCl₂ treated cells by western blotting. Untreated cells, a; 3 hr, b; 24 hr, c; 48 hr, d B: Change of p53, bcl-2, and MDM2 levels in the cells preincubated with 1 mM SIN-1 and then treated 800 μ M CoCl₂ determined by western blotting. for 47½ hr, 45 hr, 24 hr. Actin was used as loading control. Untreated cells, a; CoCl₂ 48 hr, b; SIN-1 30 min. and CoCl₂ 47½ hr, c; SIN-1 3 hr and CoCl₂ 45 hr, d; SIN-1 6 hr and CoCl₂ 42 hr, e; SIN-1 12 hr and CoCl₂ 36 hr, f; SIN-1 24 hr and CoCl₂ 24 hr, g; SIN-1 48 hr, h.

(Fig. 2). Also, the protein levels increased. After 12 hr with SIN-1 pre-treatment, p53, MDM2 and bcl-2 were increased SIN-1 treating time dependently (Fig. 4B).

Discussion

Nitric oxide (NO) is an important bioregulatory agent [5]. High concentrations of NO induce apoptotic cell death, in several cell types. However, NO can protect some cells from apoptosis through several mechanisms [9,18,20].

Several studies have shown that, although p53 level increases but p53 does not induce apoptosis [3,17,19]. Our results showed that SIN-1 did not induce apoptosis in MCF-7 cells even CoCl₂, apoptosis inducer, treatment. However, even though the apoptosis level decreased by treatment time of SIN-1, p53 level increased. p53 can be inactivated and inhibited by some proteins, MDM2, bcl-2 and p21.

The tumor suppressor protein p53 is inactivated by the cellular factor MDM2 [21]. In some tumors with wild-type

p53, other changes, such as the amplification of the MDM2 gene or overexpression of the MDM2 protein, can block p53 function and promote growth of the tumor [4]. In cells treated with SIN-1 and CoCl₂ together, MDM2 level was increased while p53 accumulation occurred. MDM2 may function by binding to p53 and inhibiting its ability to activate transcription [24].

Bcl-2 blocks NO-mediated apoptosis, while accumulation of p53 remains unperturbed. Reed has shown that NO-mediated apoptosis is blocked by bcl-2 downstream of the initial toxic insult [28] and bcl-2 overexpression delays the onset of apoptosis [25]. Bcl-2 was accumulated in SIN-1 treated cells time dependently. Overexpression of bcl-2 can block p53-mediated apoptosis. Bax binds to bcl-2, and regulates apoptosis [2,8]. But bax did not show significantly change. Bax is not an absolute prerequisite for apoptosis.

p21, the product of the p21waf/cip1 gene, is a cdk inhibitor that plays a critical role in regulating cell cycle progression [6,27]. p21 expression is independent of p53 [12]. Recently, it has been reported that p21 inhibit apoptosis [15,16,31].

Taken together, whereas the p53 level increased, it did not induce apoptosis in MCF-7 cells. MDM2 inactivated p53 and p21 prevented apoptosis independently p53. Overexpression bcl-2 inhibits apoptosis in MCF-7 cells. Therefore, the results of this study suggest that nitric oxide can protect apoptosis - related gene and promote tumor growth.

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초록 : 유방암 세포(MCF-7)에서 nitric oxide에 의한 apoptosis 억제김군하 · 노상근 · 박혜련¹ · 최원철*(부산대학교 자연과학대학 생물학과, ¹부산대학교 치과대학 구강병리학)

Nitric oxide (NO)는 세포 안의 다양한 생리학적, 병리학적 조건에서 확산, 세포 간 messenger와 같은 다양한 기능이 있으며, NO는 암세포나 macrophage 등과 같은 세포에서는 apoptosis를 유도하고, 정상세포나 내피 세포에서는 apoptosis를 억제한다고 보고되어져 있다. NO가 유방암 세포주인 MCF-7 세포에서는 apoptosis를 유도하는지 확인하기 위해 NO donor인 SIN-1을 처리하였다. SIN-1은 48시간 처리 시에도 세포 생존율에 영향을 주지 않았고, 세포주기나 성장 패턴에도 아무런 변화를 주지 않았다. 그러나 p53의 발현은 SIN-1 처리 시간에 따라 증가하였고, bcl-2, MDM2, p21의 발현도 함께 증가하였다. Bax의 발현은 SIN-1 처리 시에 변화가 없었다. MCF-7 세포에서 NO에 의한 apoptosis 억제를 보기 위하여, SIN-1을 선처리한 세포에 CoCl₂를 처리하였다. 세포에 CoCl₂만을 처리한 군에서는 확연한 apoptosis를 나타내었지만, SIN-1을 24시간 선처리한 세포에서는 apoptosis를 관찰할 수 없었다. Cobalt Chloride에 의해 감소되었던 p53, MDM2, bcl-2 발현 역시 SIN-1을 24시간 선처리한 세포에서 증가하였다. 이런 결과들은 SIN-1에 의해 발현된 MDM2가 p53의 기능을 막으며, 또한 p21과 bcl-2의 발현이 유도되어 apoptosis를 억제함을 제시한다.