NADPH oxidase inhibitor diphenyleneiodonium induces p53 expression and cell cycle arrest in several cancer cell lines

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The Diphenyleneiodonium (DPI) is widely used as an inhibitor of flavoenzymes, particularly NADPH oxidase. In this study, we investigated the effect of DPI on the cell growth progression of human colon cancer cells HCT-116 (wild-type p53), HT-29 (p53 mutant) and human breast cancer cells MCF-7 (wild-type p53). DPI treatment in cancer cells evoked a dose- and time-dependent growth inhibition, and also induced the cell cycle arrest in G2/M phase. The peak of cell population arrested in G2/M phase was observed at 12 hr after treatment of DPI. In addition, DPI significantly induced the expression of p53, which induces proapoptotic genes in response to DNA damage or irreparable cell cycle arrest, at 6 hr in DPI-stimulated cells. However, a catechol apocynin, which inhibits the assembly of NADPH oxidase, did not induce p53 expression. This suggest that p53 expression induced by DPI is not associated with the inhibition of NADPH oxidase. In conclusion, we suggest that DPI induces the expression of wild-type p53 by ROS-independent mechanism in several cancer cells, and upregulated p53 may be involved in regulatory mechanisms for growth inhibition and cell cycle arrest at G2/M phase in DPI-stimulated cells.

Key words – DPI, growth inhibition, cell cycle arrest, p53, cancer cells

Introduction

Diphenyleneiodonium (DPI) is a well-known inhibitor of flavoenzymes. It has been reported to inhibit the activity of NADPH oxidase [2], nitric oxide synthase [22], xanthine oxidase [19] and NADPH cytochrome P450 oxidoreductase [23]. Electron transport through the flavin moieties of these flavoenzymes causes reduction of DPI to its diphenyleneiodonyl radical form, followed by covalent phenylation of either the flavin or adjacent amino acid and heme groups of the proteins [3,14]. Despite its nonspecific mode of action, DPI has frequently been used to inhibit reactive oxygen species (ROS) production mediated by flavoenzymes in various types of cells [2,10,16], where the resultant oxidants are proposed to play a role in cell signaling [5].

The p53 tumor suppressor is a nuclear transcription factor, which can induce proapoptotic or suppress antiapoptotic genes in response to DNA damage or irreparable cell cycle arrest [11]. Mutational inactivation of p53 has been found to be involved in >50% of human cancers, which indicates the importance of p53 in human carcinogenesis [8]. p53 is activated in response to stimuli such as DNA damaging agents, UV, ionizing radiation, hypoxia and nucleotide deprivation. Current evidence suggests that p53 may induce cell cycle arrest or apoptosis by a multitude of molecular pathways. Reports suggest that the p53 plays as a checkpoint regulator of cell cycle and contributes to a cell cycle arrest in G1 [4,12] and G2 [1,6,7] by a multitude of molecular pathways in several cancer cells. In particular, cell cycle arrest in G2/M phase is known to mediate through inhibition of cyclin B1-cdc2 activity.

We here report the effect of DPI on the induction of wild-type p53 expression and the cell cycle arrest in several human cancer cell lines. These results suggest that DPI induces growth inhibition and the cell cycle arrest of the cells, which may be associated with the induction of p53 expression.

Materials and Methods

Materials

DPI, protease inhibitor cocktail, dimethyl sulfoxide (DMSO), trypan blue (0.4%) and propidium iodide (PI) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). 4-hydroxy-3-methoxyacetophenone (apocynin) was purchased from Calbiochem Co. (La Jolla, CA, USA). Anti-p53 and β-actin antibodies were purchased from

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Santa Cruz Biotechnology (Santa Cruz, CA, USA). The secondary HRP-conjugated antibody and ECL western blotting kits were obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Fetal bovine serum (FBS), HBSS and other tissue culture reagents were purchased from Life Technologies (Gaithersburg, MD, USA).

Cell culture

HCT-116 and HT-29 (human colon cancer cells) and MCF-7 (human breast cancer cells) were obtained from American Tissue Culture Collection (Rockville, MD, USA). Cells were cultured in DMEM (HCT-116) or RPMI 1640 (HT-29 and MCF-7) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine and antibiotics (100 U/ml of penicillin, 100 μg/ml of streptomycin) at 37°C with 10% CO_2 in air atmosphere. To maintain reproducibility, confluent cells were subcultured according to standard protocol with 0.01% trypsin-EDTA. The cells were plated and grown to 70-80% confluency for 12 hr before treatment.

Growth inhibition study

For growth inhibition analysis, cells were plated at 1×10^5 cells per 60-mm dishes and incubated for 12 hr. Cells were cultured in presence or absence of different concentrations of DPI in fresh medium supplemented with 10% FBS. After incubation of different time periods, the cells were washed with cold phosphate-buffered saline (PBS), trypsinized and centrifuged at 500×g. Cells were resuspended in medium and viable cells and dead cells were counted by the trypan blue dye exclusion method using a hemocytometer.

Cell cycle analysis by flow cytometry

Cells (1×10^6 cells/well) were cultured in 6-well plates in medium containing 10% FBS, then treated with or without DPI for different times, in control cells or in transfected cells [15]. And, cells were harvested, washed with ice-cold PBS, and fixed with 70% ethanol/PBS at 4°C. Then, the fixed cells were incubated with 0.5 ml PBS containing 20 μg/ml RNase A for 30 min at 37°C, and stained with 50 μg/ml PI for 30 min in the dark at room temperature, and finally analyzed by a FACSCalibur™ flow cytometer (Becton Dickinson, San Jose, CA, USA). A minimum of 1×10^4 cells per sample was evaluated and the percentage of cell distributions at each phase of cell cycle was further calculated by CellQuest and ModFit (Becton Dickinson, San Jose, CA, USA) software programs [24].

Western blot analysis

Equivalent amounts of total protein were loaded onto 10% SDS/PAGE. The gels were transferred to nitrocellulose membrane using an electroblotting apparatus (Bio-Rad, Richmond, CA, USA) and reacted with each antibody according to standard methods. Bound immunocomplexes were visualized on X-ray film by ECL reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA). β-actin was used as an internal control to monitor equal protein sample loading.

Statistical analysis

Three independent assays were performed. Statistical values were expressed as the means ± standard deviation (S.D.) of the means obtained from each independent experiment. The results of the experimental and control groups were tested for statistical significance by a one-tailed Student's *t* test.

Results and Discussion

DPI induces growth inhibition and cell cycle arrest in several cancer cells

Although DPI, as an NADPH oxidase inhibitor, can be expected to decrease the cellular generation of ROS, recent results about this activity are controversial. Both stimulation and inhibition of ROS generation have been reported [9,10,16,18]. Therefore, we investigated whether DPI could contribute to the generation of intracellular ROS in HCT-116 cells, which express functional wild-type p53. DPI did not induce the generation of ROS at early time points, 0.5 to 6 hrs (data not shown). We analyzed whether DPI evokes the induction of p53 expression by inhibition of ROS generation. Initial experiments were designed to determine the effect of DPI on cell growth of HCT-116, HT-29 (p53 mutant) and MCF-7 (wild-type p53) cells. Cells were treated with various concentrations of DPI (1, 10 and 20 μM) and collected at different time point after treatment. The cells did not show a significant evidence of decreased cellular viability after 6 and 12 hrs with DPI (data not shown). However, a marked reduction in viable cell counts was observed after 24 hr of treatments with dose-dependent concentrations of DPI (Fig. 1). This suggests that exposure

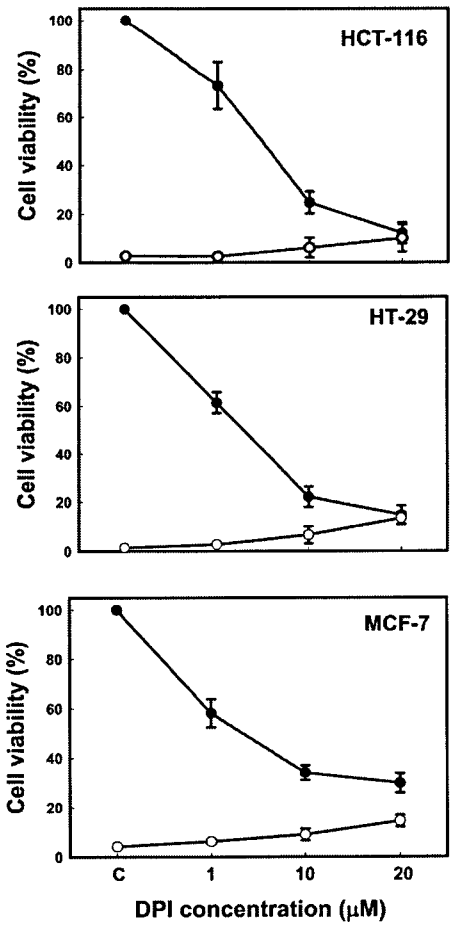

Fig. 1. Effect of DPI on cell viability in several human cancer cell lines. Cells were plated at 1×10^5 cells per 60-mm dishes, incubated for 12 hr and treated with different concentrations of DPI for 24 hr. After treatment, the cells were collected, trypsinized and washed with PBS, at each time point. Then, the viable cells were scored by hemocytometer counts of trypan blue-excluding cells. Values were determined as a percentage to total cells (live cells, ●; dead cells, ○). Three independent experiments were performed and data shown are means ± S.D. obtained from triplicate of each experiment.

of DPI to cells results in a marked decrease of cell proliferation without significant toxicity for necrotic cell death. To determine whether growth inhibition induced by DPI is associated with arrest of cells in a particular phase, we analyzed cell distributions at each phase of cell cycle by flow cytometry. In untreated cells, 35-45% of the cells were in the G1 phase and 25-30% were in G2/M phase. As expected, DPI resulted in a significant accumulation of cells in G2/M phase, with a dramatic loss of cells in G1 and S phase. The peak of cell population arrested in G2/M phase was observed at 12 hr after treatment of DPI (Fig. 2).

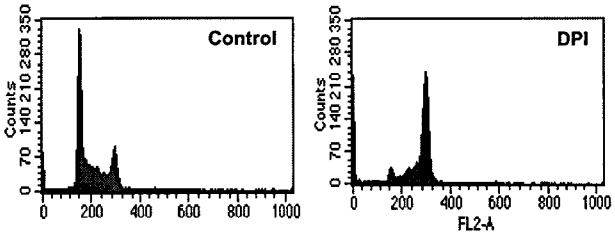

Fig. 2. Effects of DPI on cell cycle arrest in human colon cancer HCT-116 cells. Cells were plated at 1×10^6 cells per 60-mm dishes, incubated and treated with 10 μM DPI for 12 hr. After incubation, the cells were collected, stained and analyzed for cell distributions at each phase of cell cycle by flow cytometry, as described in Materials and Methods.

DPI induces the expression of p53 at early time point in cells containing wild-type p53 but not apocynin

Next, to determine whether growth inhibition and apoptosis induced by DPI is correlated with p53 expression, we analyzed the amount of expressed p53 by western blotting. Surprisingly, DPI markedly induced the expression of p53 in a dose-dependent manner (Fig. 3A). Phosphorylation of p53 has been shown to influence p53 activity under physiological

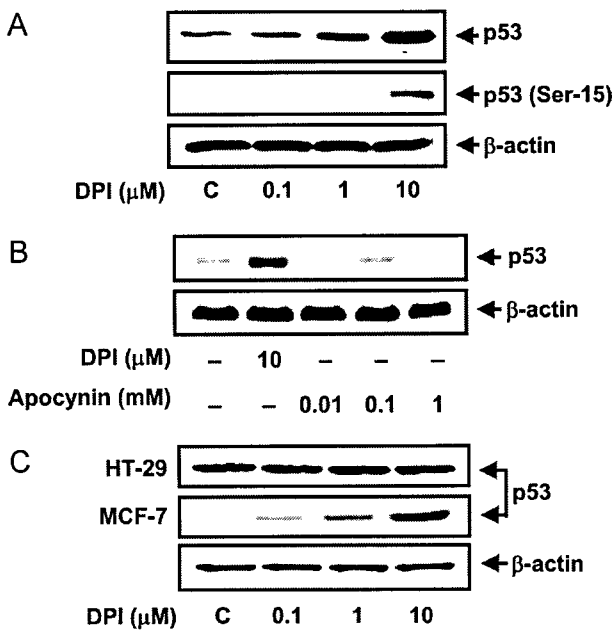

Fig. 3. Effect of DPI and apocynin on the expression of p53 in several cancer cells. Cells were treated with 10 μM DPI for 24 hr. Whole cell extracts were analyzed by western blotting using corresponding antibodies. (A) Full-length p53 and phospho-p53 (Ser-15). (B) Effect of apocynin. (C) Effect of DPI on p53 expression in HT-29 and MCF-7 cells. β-actin was used as an internal control to monitor equal protein loading.

and stress conditions. As described previously [17], phospho-p53 at Ser-15 physically interacts with bcl-2 and bcl-xL in mitochondria and precedes cytochrome c release and mitochondrial membrane potential (MMP) reduction. Therefore, we assessed levels of phospho-p53 at Ser-15 by western blotting. As expected, DPI caused phosphorylation of p53 at Ser-15 in HCT-116 cells. However, a catechol apocynin, which inhibits the assembly of NADPH oxidase, did not (Fig. 3B). We further investigated the effect of DPI on p53 expression by DPI in another cancer cell lines HT-29 and MCF-7 containing p53 mutant and wild-type p53, respectively. As shown in Fig. 3C, DPI only induced p53 expression in MCF-7 cells, which express functional wild-type p53. These results suggest that p53 expression induced by DPI is not associated with the inhibition of NADPH oxidase We also examined the exact time point on the p53 expression in DPI-stimulated cells. Interestingly, DPI markedly induced the expression of p53 at 6 hr in DPI-stimulated HCT-116 cells (Fig. 4). This suggests that upregulated p53 may be involved in regulatory mechanisms for growth inhibition and cell cycle arrest at G2/M phase in DPI-stimulated cells. This report demonstrates that DPI induces the expression of p53 by ROS-independent mechanism in several cancer cells, and renders cells sensitive to the cell cycle arrest by induction of p53 expression. This does fit with recent work in other cancer cells from other investigators demonstrating that DPI affected selective and irreversible inhibition of cell cycle [20]. DPI is nonspecific flavin binder. Moreover, experimental evidence suggests that the action of DPI and other iodonium-containing compounds are not restricted to flavoenzymes only [13,21,25]. In conclusion, the results presented in this report demonstrate that DPI induces growth inhibition and the cell cycle arrest of several human cancer cells, which may be associated with the induction of p53 expression. Although much remains to be clarified regarding the action of DPI on the cell cycle, the ability of DPI to induce the cell cycle arrest and p53 expression has exposed a potent and novel anticancer activity that may have significant implications for the targeting of cancerous cells.

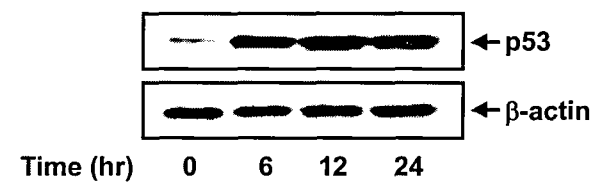

Fig. 4. Time course of p53 expression induced by DPI. Cells were treated with 10 μM DPI for 0, 6, 12 and 24 hrs. Whole cell extracts were analyzed by western blotting using a antibody for p53. β-Actin was used as an internal control to monitor equal protein loading.

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초록 : NADPH oxidase 저해제인 diphenyleneiodonium의 p53 발현 및 암세포의 성장억제에 대한 연구

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Diphenyleneiodonium (DPI)는 NADPH oxidase 같은 flavoenzymes의 저해제로써 널리 사용되고 있다. 본 연구에서는 인간 대장암 세포주 HCT-116 (wild-type p53)와 HT-29 (p53 mutant) 및 인간 유방암 세포주인 MCF-7 (wild-type p53)의 세포성장 과정에서의 DPI의 효과를 살펴보았다. DPI는 농도 및 시간 의존적으로 암세포주의 성장을 막았으며 G2/M phase에서 cell cycle arrest를 일으켰다. Cell cycle arrest의 가장 높은 값은 DPI 처리 후 12 시간에서 관찰할 수 있었다. 한편 DPI는 아폽토시스 그리고 cell cycle arrest에 관여하는 유전자 발현에 관여하는 p53의 표현을 크게 증가시켰으며, 이는 DPI 처리 후 6 시간 후 부터 관찰할 수 있었다. 그러나 NADPH oxidase의 조합을 억제하는 catechol 계인 apocynin은 p53의 발현을 유도하지 못하였다. 이것은 DPI에 의해 유도되는 p53의 발현증가는 NADPH oxidase 활성의 저해와 관련되어 있지 않다는 것을 의미한다. 결론적으로 DPI는 HCT-116, HCT-15 및 MCF-7 암세포주에서 ROS에 비의존적으로 wild-type p53 발현의 증가를 유도하며, 이 증가된 p53은 DPI에 의해 유도되는 성장 억제 및 G2/M phase에서의 cell cycle arrset 과정의 조절기전에 관여한다는 것을 시사한다.