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Master's Thesis

The mechanism of P3-mediated ROS regulation

Graduate School of Chosun University

Department of Bio-Materials Engineering

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Advisor: Professor Ho Jin You

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CONTENTS

ABSTRACT ····· 1

I. INTRODUCTION ····· 3

II. MATERIALS AND METHODS

1. Cell culture ····· 8
2. Stable Cell lines ····· 8
3. The cell treatment ····· 9
4. Construction of Adenoviral vector ····· 9
5. Western Blot ····· 9
6. Coomassie Blue Staining ····· 10
7. Immunoprecipitation ····· 10
8. Immunofluoressence Analysis ····· 10
9. Catalase Activity Assay ····· 11
10. Measurement of intracellular ROS ····· 11
11. Apoptosis by PI staining ····· 11

III. RESULTS

1. Binding between P3 and Catalase ····· 12
2. Effect of P3 on Catalase activity ····· 16
3. Role of catalase in P3-induced apoptosis ····· 24

IV. DISCUSSION ····· 27

V. REFERENCES ····· 29

KOREAN ABSTRACT ····· 32

ABSTRACT

The mechanism of P3-mediated ROS regulation

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The p53, tumor suppressor protein, is activated in response to multiple forms of stress, and is a key regulator of cell cycle, DNA repair and apoptosis. Widespread genomic alterations of p53 are frequently found in cancers. Increased production of reactive oxygen species (ROS) has been recognized as a critical factor in cancer. Several genes are predicted to encode proteins that can modulate the levels of intracellular reactive oxygen species (ROS). Of particular interest is the p53-inducible gene 3 (P3), which contains a p53 consensus binding site in its promoter region and shares significant homology with oxidoreductases from several species. Normal aerobic metabolism is associated with the production of ROS including superoxides, hydrogen peroxide hydroxyl radicals, and nitric oxide. Already in 1991, it has been shown that hydrogen peroxide is able to induce apoptosis, which is prevent by catalase. Catalase mediates the predominant enzymatic mechanisms regulating intracellular H_2O_2 levels. The tetrameric catalase converts H_2O_2 to H_2O and O_2 in peroxisomes. All of these findings support a critical role of ROS accumulation establish in P3-dependent cell fates decision and ROS inducer can collaborate with P3 to influence these fate decisions.

To detect the mechanism of P3, the binding between catalase and P3 were checked using *in-vitro* and *in-vivo* co-immunoprecipitation. Catalase activity was analyzed after P3 over-expression in MCF7, SW480, NCI H460, NCI H1299, HCT116p53$^{-/-}$ and SaoS-2 cell lines. We found that the catalase activity was downregulated while the ROS level was increased in above cell lines except NCI H460 which was catalase deficient cell line. In P3 over-expressed NCI H460 cell line, after Ad Catalase infection, ROS level were increased. After over-express of P3 in

HCT116p53$^{-/-}$cell line, these cells were stimulated by UV irradiation. And after 48h, the catalase activity, ROS levels and apoptotic cells were checked. From our results it showed that when the catalase activity were decreased by P3 over-expression, ROS levels were also increased, it lead to apoptosis consequently.

The results demonstrate that P3-mediated ROS regulation for the execution of apoptosis in human cells is through the binding between P3 and catalase protein. This direct binding influence catalase activity and decreased catalase activity is responsible for increased ROS levels and high percentage of apoptotic cell death consequently.

I. INTRODUCTION

1. P3

P53 (also known as tumor protein 53 (TP53)), one of the first tumor suppressor genes to be identified, remains the most frequent target for genetic alteration identified in human cancers. The principal function of p53 appears to be in mediating a response to DNA damage, thereby preventing accumulation of potentially oncogenic mutations and genomic instability. This role of p53, as guardian of the genome, provides the basis for its tumor-suppressive activities [1]. It can be activated by many types of "danger signals", such as cell stress and DNA damage, and can trigger several crucial radiation-, drug-, or carcinogen-induced DNA damage, oncogenic activation, hypoxia and low ribonucleotide pools. These conditions may nurture tumor initiation [2]. Several downstream p53 target genes have been identified. Some of them (e.g. p21, GADD45) play a role in cell cycle control, inducing G1 and G2 arrest in the presence of DNA damage. Others are involved in the induction of apoptosis (e.g. Bax, IGF-BP3, P3), or negatively regulate p53 activity and stability (MDM2).

P3 (Tumor protein p53 inducible protein 3, also known as TP53I3) is one of the p53 protein targets, and is used as long-lived proapoptotic marker. P3 was discovered along with twelve other proteins in a serial analysis of gene expression studies designed to determine gene induced by p53 before the apoptosis onset [3].

The protein encoded by P3 is similar to oxidoreductases, which are enzymes involved in cellular responses to oxidative stresses and irradiation. This gene is induced by the tumor suppressor p53 and is thought to be involved in p53-mediated cell death. It contains a p53 consensus binding site in its promoter region and a downstream pentanucleotide microsatellite sequence. P53 has been shown to transcriptionally activate this gene by interacting with the downstream pentanucleotide microsatellite sequence. The microsatellite is polymorphic, with a varying number of pentanucleotide repeats directly correlated with the extent of transcriptional activation by p53. It has been suggested that the microsatellite polymorphism may be associated with differential susceptibility to cancer. At least two transcript variants encoding the same protein have been found for this gene [1].

P3 protein was localized to the cytoplasm and induced in primary, non-transformed, and transformed cell culture after exposure to genotoxic agents. The induction of P3 was p53 dependent and occurred with delayed kinetics as compared with other p53 downstream targets,

such as p21 and MDM2. Using a p53-inducible cell model system, in which p53-mediate growth arrest is reversible, we found that P3 levels were increased during p53-mediate growth arrest [4]. There is some evidence to suggest that the P3 protein is involved in the generation of reactive oxygen species (ROS), which are important downstream mediators of the p53-dependent apoptotic response. First, P3 expression precedes the appearance of ROS in p53-induced apoptosis. Second, P3 shares sequence similarity with numerous NAD(P)H quinine oxidoreductases shown to be potent inducers of ROS. Third, certain p53 mutants capable of inducing cell arrest but not apoptosis retain the ability to activate target genes such as the cyclin-dependent kinase inhibitor p21, but not P3. However, because P3 expression alone is insufficient to cause apoptosis, it is suspected that many factors cooperate to cause ROS-dependent cell death. At this time, the exact role of P3 in p53-dependent apoptosis remains to be determined.

2. Reactive oxygen species (ROS)

ROS (reactive oxygen species) are implicated in variety of different pathologies, such as diabetes and neurodengenerative diseases (e.g. Alzheimer's disease and Parkinson's disease). With regard to tumor development, ROS have been considered as DNA-damaging agents that increase the mutation rate and promote oncogenic transformation [5]. The term ROS is used for short-lived diffusible entities such as hydroxyl (·OH), alkoxyl (·RO) or peroxyl (·ROO) radicals and for some radical species of medium lifetime such as superoxide (O_2·) or nitroxyl radical (NO·). It also includes the non-radical hydrogen peroxide (H_2O_2), organic hydroperoxides (ROOH) and hypochlorous acid (HOCl). ROS are generated by inflammatory cells, which accumulate in both allergic [6] and non-allergic [7] inflammations. ROS have destructive actions on both DNA and proteins. It is therefore not surprising that ROS are part of the defense mechanism against bacteria. Antimicrobial action must be very specific since ROS may also destroy tissues, a scenario that actually occurs under strong inflammatory conditions [8, 9]. However, antioxidants (reduced glutathione, catalase, superoxide dismutase) usually prevent tissue damage under normal conditions. The generation of ROS in antimicrobial defense mechanisms mainly occurs in phagocytes due to the activation of the NADPH oxidase and is also called respiratory burst [10]. The respiratory burst is often used as read-out in functional assay using phagocytes [11, 12]. There is growing evidence that ROS may also acts as chemical messengers, besides its role as destructive agents. For instance, ROS appear to be involved in receptor-mediated signaling pathway [13-15] and transcriptional activation [16]. In this article, we will focus on the role of ROS in the regulation of apoptosis.

3. Apoptosis induction by ROS

Already in 1991, it has been shown that hydrogen peroxide is able to induce apoptosis, which is prevented by catalase [17]. Besides catalase, high intracellular glutathione levels oppose the apoptosis striggered by ROS. Since then many authors have shown that ROS can induce apoptosis in many different cell systems. For instance H_2O_2 induced apoptosis in neotrophils, which can be prevented by catalase[18]. Catalase also prevented spontaneous neutrophil apoptosis, suggesting that the generation of H_2O_2 might be one important trigger mechanism responsible for the short life-span of mature neutrophils. In addition, augmented intracellular glutathione levels prevented Fas receptor-mediate apoptosis in these cells [19].

Similar studies have been performed in eosinophils. When ROS production was generated by sodium arsenite, induction of eosinophil apoptosis was observed, whereas higher concentrations had no effect [20]. Induction of apoptosis using low concentrations was also observed by using phenylarsine oxide in both neutrophils and eosinophils [21]. This study suggested that apoptosis is regulated by tyrosine phosphorylation and it is possible that the phosphorylation-state of at least some apoptosis regulating proteins are influenced by ROS.

Taken together, there is growing evidence that ROS are important for the induction of apoptosis in inflammatory but also other cells (not discussed). The functional importance of ROS generation for the activation of death mechanisms has been demonstrated by inhibitior studies. There are numerous examples of the inhibition of apoptosis through antioxidative drugs or enzymes.

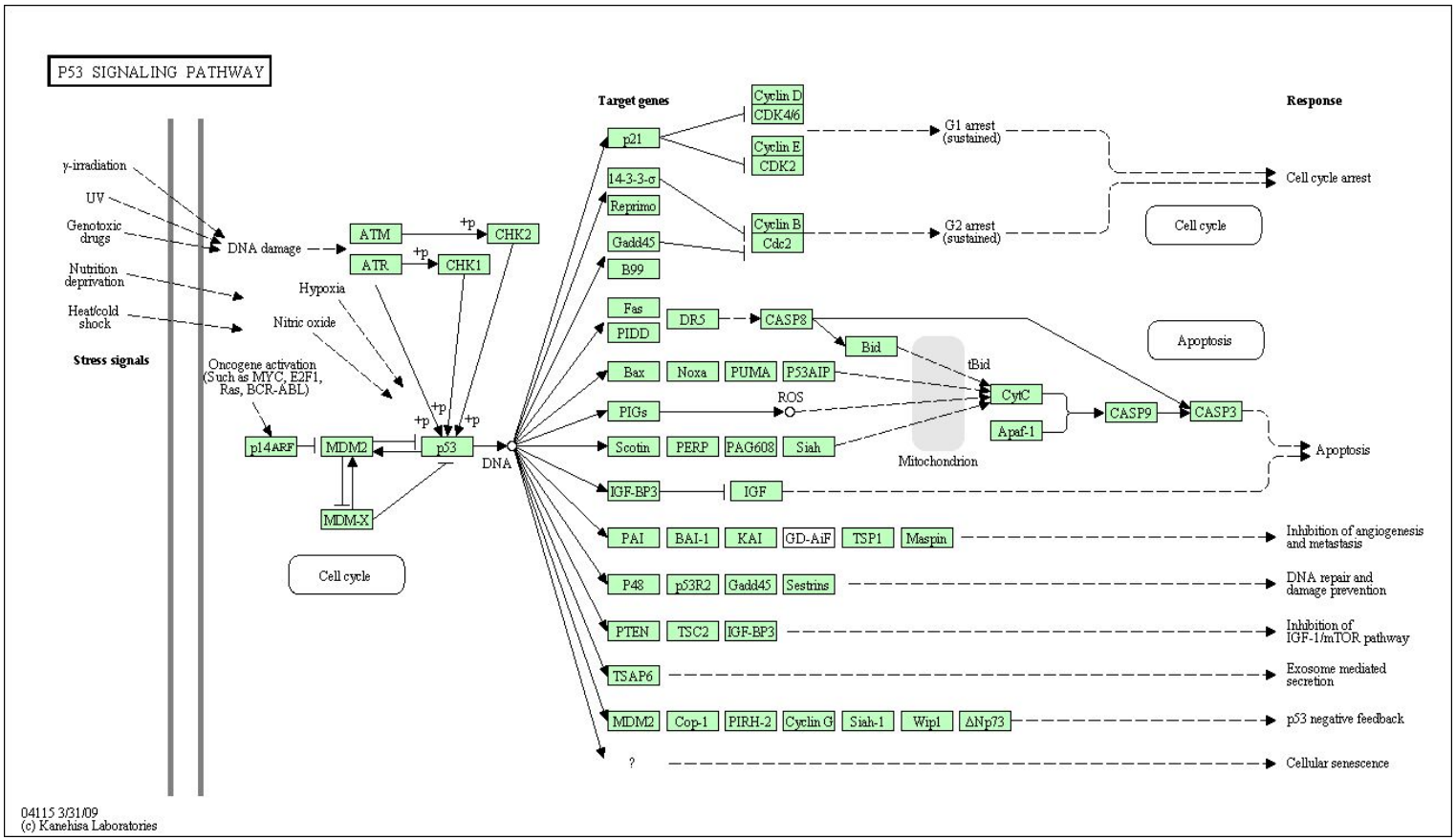

Fig.1. P3 pathway in apoptosis induction.

4. Purpose of study

In the view of the implication of ROS in apoptosis and the capacity of P3 to elevate ROS levels, the aims of the current study were to find the mechanism of P3-mediate ROS regulation. To elucidate the function of P3 in regulation of ROS-mediated apoptosis, relationship between P3 and catalase was analyzed.

II. MATERIALS AND METHODS

1. Cell culture

The human colon carcinoma cell line RKO (ATCC, CRL-2577) and osteosacoma cell line U2OS (ATCC, HTB-96) containing wilp-type p53 were maintained in Eagle's minimum essential medium (EMEM, Invitrogen corporation) and McCoy's 5A (Mediatech, Inc), respectively, supplemented with 10% fetal bovine serum (FBS, Cambrex), 100units/ml of penicillin, and 100mg/ml of streptomycin (Penicillin-Streptomycin, Invitrogen corporation). The human lung carcinoma cell line NCI H1299 (ATCC, CRL-5803) and NCI H460 (ATCC, HTB-177) and human colon colorectal adenocarcinoma SW480 (ATCC, CCL-228) were cultured in RPMI1640 (Invitrogen corporation) supplemented with 10% FBS, 100units/ml of penicillin, and 100mg/ml of streptomycin. The human adenocarcinoma MCF7 (ATCC, HTB-22) was cultured in EMEM (Invitrogen corporation) supplement with 10% FBS, 100units/ml of penicillin, and 100mg/ml of streptomycin. The human colon colorectal carcinoma HCT116p53 (ATCC, CCL-247) was cultured in IMDM (Invitrogen corporation) supplemented with 10% FBS, 100units/ml of penicillin, and 100mg/ml of streptomycin. The human bone osteosarcoma SaoS-2 (ATCC, HTB-85) was maintained in McCoy's 5A (mediatech, Inc), supplemented with 15% FBS, 100units/ml of penicillin, and 100mg/ml of streptomycin. These cell lines were seeded in 100cm^2 tissue culture plates (IWAKI), grown in 37^0C, 5% CO_2 incubator and checked by microscope everyday.

2. Stable cell lines

Full-length human P3 cDNA was directly cloned into the pcDNA 3.1 TOPO-V5-His using specific primer: forward, 5'-ATGTTAGCCGTGCACTTTGACAA-3', and reverse, 5'-TCACTGGGGCAGTTCCAGGAC-3' for P3. The result plasmids were transfected into HCT116p53-/- using Lipofectamine 2000 (Invitrogen). pcDNA3.1 TOPO-V5-His LacZ was used as a control. The cells were cultured in IMDM medium supplemented with 10% FBS and 100units/ml of penicillin, and 100mg/ml of streptomycin. After 24h, hygromycin antibiotic (Invivogen) was added to the culture medium at a concentration of 800μg/ml to select hygromycin-resistant colonies. After 4 weeks of culture with the change of the hygromycin-containing medium every 3 days, hygromycin-resistant colonies were isolated and confirmed by western blot.

3. The cell treatment

The cells were stimulated by UV irradiation 20J/cm^2 to undergo apoptosis in HCT116p53$^{-/-}$ (Mock) and HCT116p53$^{-/-}$ (P3) cell lines. After 48h UV irradiation, cells were examined. NCI H460 cell line was examined at 48h after Ad GFP and Ad Catalase infection.

4. Construction of Adenoviral vector

The Catalase cDNA was cloned into an Ad/CMV/V5-DEST vector (Invitrogen, Carsbad, CA) after confirming the DNA sequence. Ad/CMV/V5-DEST GFP was used as control vector. The Ad GFP and Ad Catalase plasmid DNAs were linearized with PacI and purified and transfected to HEK293T cells using Lipofectamine 2000 (Invitrogen). For virus collection, the cells were lysed with three consecutive freeze-thaw cycles, and the virus was collected from the supernatant. The virus titer was ~1x10^{7}p.f.u. /ml, which was determined using an end-point dilution assay.

5. Western blot

Cells were washed with phosphate-buffered saline (PBS) and lysed with NP-40 lysis buffer (NP-40 lysis buffer added protease Inhibitor Cocktail tablet EDTA free (Roche)) on ice for 10minutes. NP-40 buffer (pH=8.0) consists of 50mM Tris (Amresco), 150mM NaCl (Duchefa Biochemie) and 1% NP-40 (USB corporation Cleveland, OH USA). After incubation, extracts were mixed for 5 minutes using micro tube mixer (Tomy MT360) and centrifuge at 13,000rpm for 15-30 minutes. The supernantant was diluted with 5X SDS-sample buffer that contain 125mM Tris (Amresco), 960mM Glycine (Amresco), 0.5% SDS (Sodium Dodecyl Sulfate, Amresco) and boilded. After cellular protein concentrations were determined using the dye-binding microassay (Bio-Rad, Hercules, CA), samples were then loaded 10μg, 20μ, or 50μg per lane and electrophoresed on 10% or 15% SDS polyacrylamide gels. The proteins were blotted onto Polyvinylidene Fluoride transfer membranes (BiotraceTM PVDF, Pall Corporation). After electroblotting, the membranes were then blocked by 5% skim milk in Tris buffer saline containing 0.05% Tween-20 (TBST, 10mM Tris-HCl, pH 7.4, 150mM NaCl, 0.1% Tween-20) at room temperature for 2h. The membranes were rinsed with TBS-T and then incubated with appropriate primary antibodies in TBST at 4^{0}C overnight.

Immunoblotting was carried out with Tubulin-α Ab-2 antibody from Clone DM1A, NeoMarkers; Catalase and P3 from Santa Cruz Biotechnology; anti-Catalase from LabFrontier (Seoul, Korea). We followed manufacture's protocols for dilution of all primary antibodies.

The membranes were then washed and incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody (1:4,000) in blocking buffer for 2 hours at room temperature, and washed again. The blotted proteins were developed using an enhanced chemiluminescent detection system (iNtRON, Biotech, Seoul, Korea).

6. Coomassie Blue Staining

Protein were loaded onto SDS-PAGE gel and run by electrophoresis until spreading the whole gel. Then gel was fixed in 10 volumes of 1X fixing solution (50% methanol and 10% acetic acid) for 30 minutes at room temperature. After discarding the fixing solution, gel was stained in 10 volumes of Coomassie Blue staining solution (0.1% Coomassie Brilliant Blue R-250, 50% methanol and 10% acetic acid) for 1h at room temperature. And then the gel was destained in 10 volumes of 1X destaining solution (40% methanol and 10% acetic acid) for 30 minutes at room temperature or until background of the gel is totally destained. After that gel was soaked in 1-2% glycerol in distil-water for 15minutes, the gel was developed using photograph.

7. Immunoprecipitation

Cells were washed with phosphate-buffered saline (PBS) and lysed at room temperature for 5minutes with mixing in NP-40 lysis buffer (50mM Tris-HCl, 150mM NaCl, 1% NP40, pH 8.0) supplemented with Protease Inhibitor Cocktail Tablet (Roche)). After incubation, total cell extracts were sonicated and centrifuged at 13,000rpm at 4^0C for 20 minutes. 1mg of protein extraction were precleared with G-agarose for 30 minutes and then 1st-antibody or normal IgG was added and then incubated overnight at 4^0C with rotation. After incubation with G-agarose for 30 minutes, precipitated complexes were gently washed three times with RIPA buffer (25mM Tris-HCl, 150mM NaCl, 5mM $MgCl_2(.6H_2O)$ 0.5% NP-40, 1mM DTT, 5% glycerol, pH 7.2), NP-40 and Tris buffer (Tris-HCl 0.1M) supplemented with mini-protease inhibitor cocktail without EDTA. Samples were resuspended in 5X-SDS samples buffer and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), then transferred onto polyvinylidene difluoride membrane, and immunoblotted.

8. Immunofluorescence Analysis

Cells grown on cover slips were rinsed with PBS and fixed with 100% methanol for 15 minutes at room temperature. After being washed with PBS, the cover slips were blocked in 5% bovine serum albumin (BSA) in PBS for 1h at RT then incubated overnight at 4^0C with the following primary antibody P3 (H-300, Santa Cruz) and Catalase (N-17, Santa Cruz), with ratio 1:50. After washing with PBS three times, secondary antibodies labled with FITC-conjugated chicken anti-rabbit IgG and Rhodamin-conjugated chicken anti-goat IgG (Molecular Probes)

were added (1:100) and incubated at RT for 1h. After washing, slides were mounted with containing DAPI. Images were acquired with confocal microscope (Zeiss LSM 510 Meta; Carl Zeiss, Germany).

9. Catalase activity assay

The standard procedure was followed as Amplex Red Catalase Assay KIT protocol and prepared stock solution according to the manufacture's instruction. The catalase activity was assayed by adding H_2O_2 solution (prepared following the manufacture's instruction) for 30 minutes at room-temperature in the dark. And then, the second phase of the reaction began by adding the Amplex Red/HRP working solution and the mixture was incubated for 30 minutes or longer at 37^0C in the dark. The absorbance was measured in a microplate reader at 560nm and analyzed.

10. Measurement of intracellular ROS

The intracellular production of ROS was assayed using the 5-(and-6)-chloromethyl-2', 7'-diclorodihydrofluescein diacetate, acetyl ester (CM-H_2DCFDA) probe (Invitrogen, Molecular Probes). CM-H_2DCFDA is oxidized by ROS to the highly fluorescent CM-DCF compound. Cells were incubated with CM-H_2DCFDA (5μM) for 30 minutes in the dark at 37^0C. After two washes with PBS, trypsin detached cells were centrifuged and resuspended by PBS containing 0.2% formalin and analyzed by flow cytometry (FACS Calibur, BD Biosciences). Ten-thousand individual data points were collected for each sample.

11. Apoptosis by PI staining

The floating and trypsin-detached cells were collected and washed once with ice-cold PBS, followed by fixing in 100% cold ethanol for 30 minutes at 4^0C or overnight at -20^0C. Then, these cells were washed in PBS and stained with PI (propidium iodide 50μg/ml), RNAase A (50μg/ml), and 0.05% Triton X-100. The DNA contents of these cells were analyzed by CELLQuest software (FACSCalibur, BD Biosciences). At least 10,000 events were analyzed, and the percentage of cells in sub-G1 population was calculated. Aggregates of cell debris at the origin of histogram were excluded from the sub-G1 cell.

III. RESULTS

1. Binding between P3 and catalase

To determine the molecular weight of catalase and P3, catalase and P3 protein were run electrophoresis and stained with Coomassie Blue. The results were shown in Figure 2A.

To study the binding between catalase and P3, we investigated the interaction *in-vitro*. We used P3 (H-300, Santa Cruz) as first antibody in co-immunoprecipitation (Co-IP). By immunoblotting using anti-catalase antibody, we could see the band which come from the binding of catalase and P3, control IgG sample completely did not show any band (Fig.2B.).

To check the binding ability between two proteins *in-vivo*, RKO cell line was exposed to UV irradiation for 24h, and then cell lysates were examined by using P3 or catalase as 1st-antibody in turn for IP and then western blot were conducted.

The result showed that binding between P3 and catalase was confirmed by co-IP. Furthermore, cell exposed to UV indicated stronger interaction than un-treated one. Control IgG lane showed no bands (Fig.2C.). Thus, in both *in-vitro* and *in-vivo* conditions, catalase and P3 showed the interaction ability, and the binding is enhanced after UV irradiation.

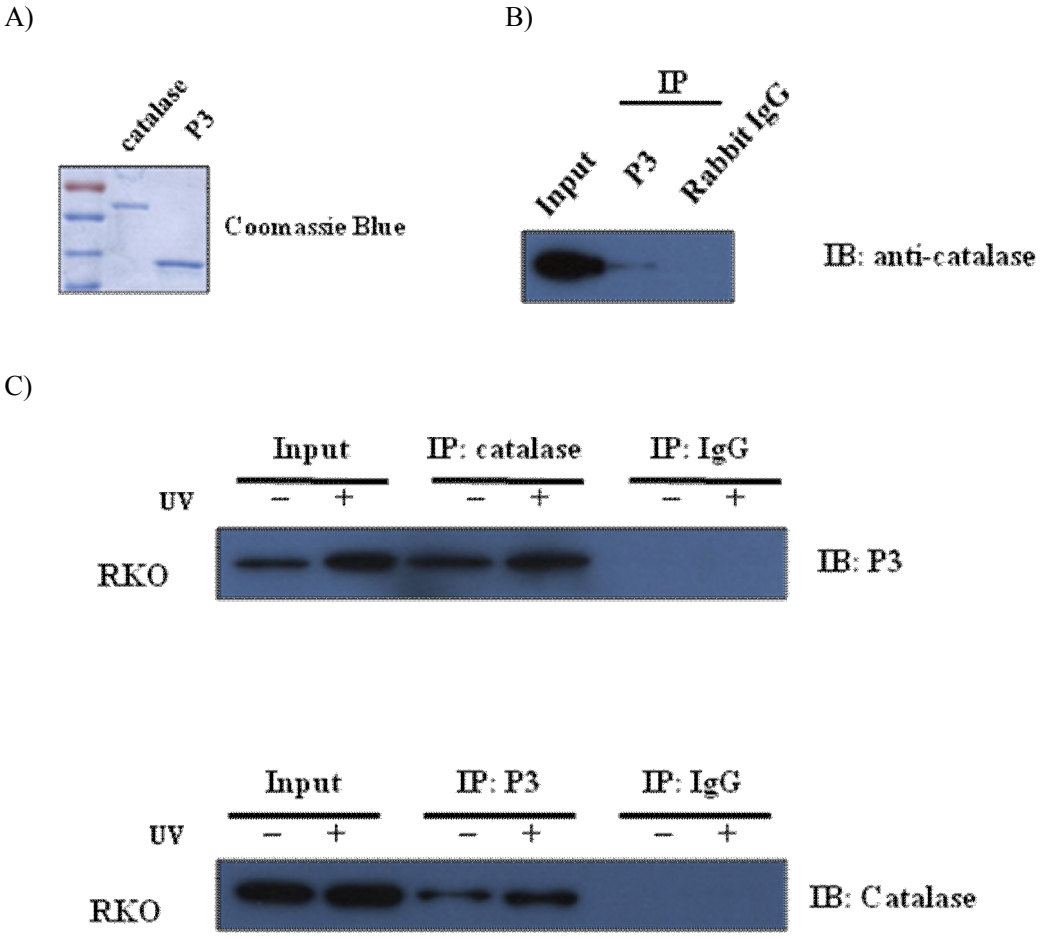

Fig.2. The *in-vitro* and *in-vivo* binding between P3 and catalase

A. Molecular weight of two proteins Catalase and P3 were determined using Coomassie blue staining.

B. Co-IP result showed the binding between P3 and Catalase proteins *in-vitro.*

C. The direct binding between Catalase and P3 in RKO cell line after UV irradiation.

To determine the localization of two proteins, RKO cell line was used for immunofluorescence analysis under UV irradiation or untreated condition.

The result showed that P3 appeared as green color, catalase as red color and the merged showed yellow color, DAPI (blue) as control of nucleus staining (Fig.3.). After UV exposure, those merged image in cytosol appeared stronger than un-treated samples. This result support evidence of co-localization, and the co-localization in cytosol under UV exposure was increased.

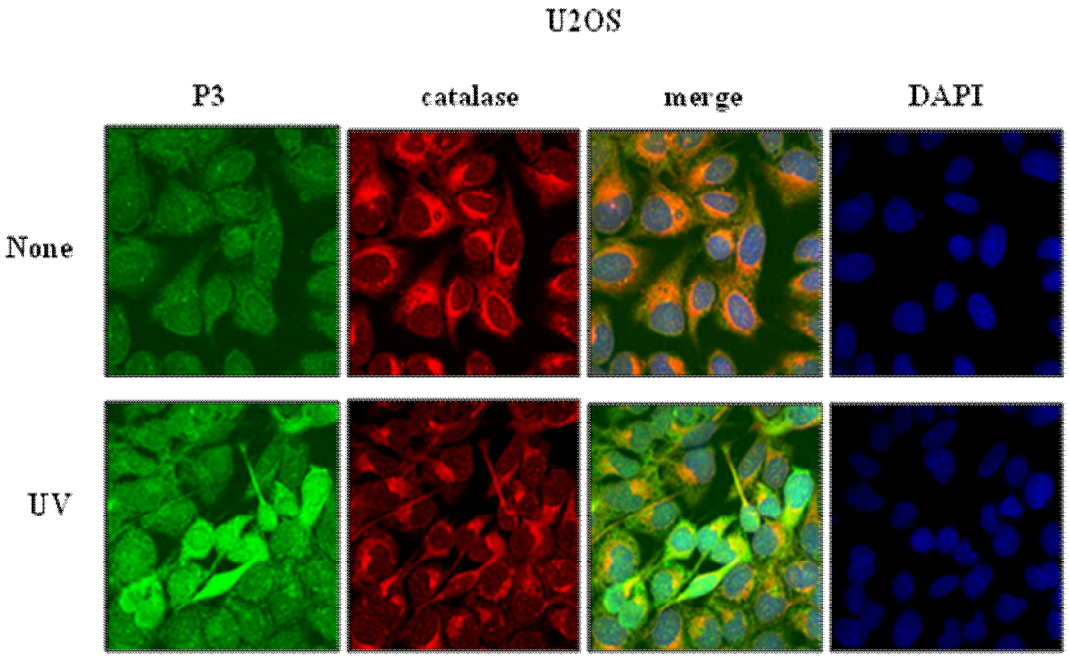

Fig.3. Co-localization of P3 and Catalase after UV irradiation

2. Effect of P3 on catalase activity

We sought to determine how P3 can effect on catalase activity leading to ROS-regulated apoptosis. To study the effect of protein P3 on catalase activity *in-vitro* we used purified protein P3. We tried to analyzed catalase activity according to concentration of P3: 0, 1, 10, 100, 1000ng.

As shown in Fig.4., the catalase activity was decreased about 20-30% in dose-dependent manner from 1 to 1000ng. Our results indicated that catalase activity was regulated by P3 through its binding ability.

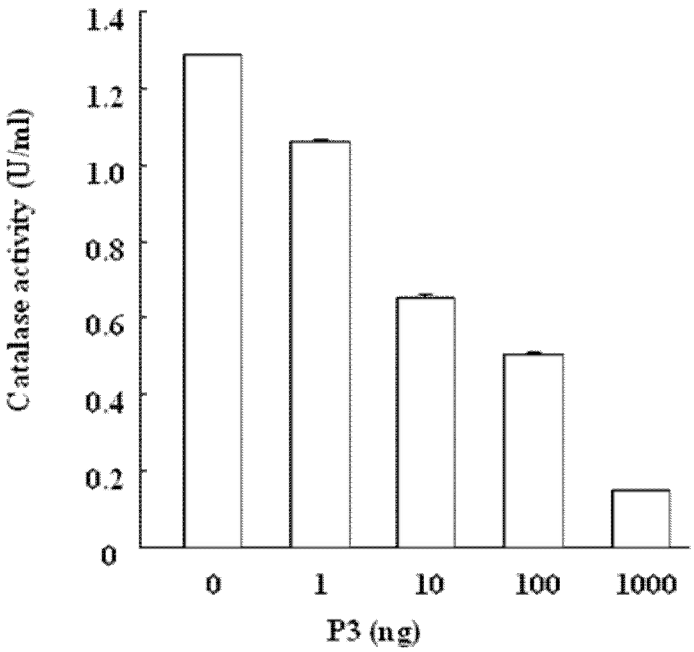

Fig.4. The effects of P3 on *in-vitro* catalase activity

To re-confirm the effect of P3 on catalase activity *in-vivo*, we transfected TOPO P3 (expression vector) or TOPO LacZ (control vector) into 6 different cell lines: MCF7, SW480, NCI H460, NCI H1299, HCT116p53$^{-/-}$ and SaoS-2. After 48h transfection, catalase activity was determined. All of cell lines except NCI H460 showed decreased level of catalase activity (Fig.5A.)

After over-expression of P3 in MCF7, SW480, NCI H460, NCI H1299, HCT116p53$^{-/-}$ and SaoS-2 cell lines, western blotting was conducted. Catalase expression pattern was different in NCI H460 cell line (Fig.5B.).

From those results, we postulated that because the NCI H460 is catalase deficient, although over-expression of P3, there was no effect on catalase activity. We elucidated the role of P3 in catalase activity regulation in NCI H460 cell line.

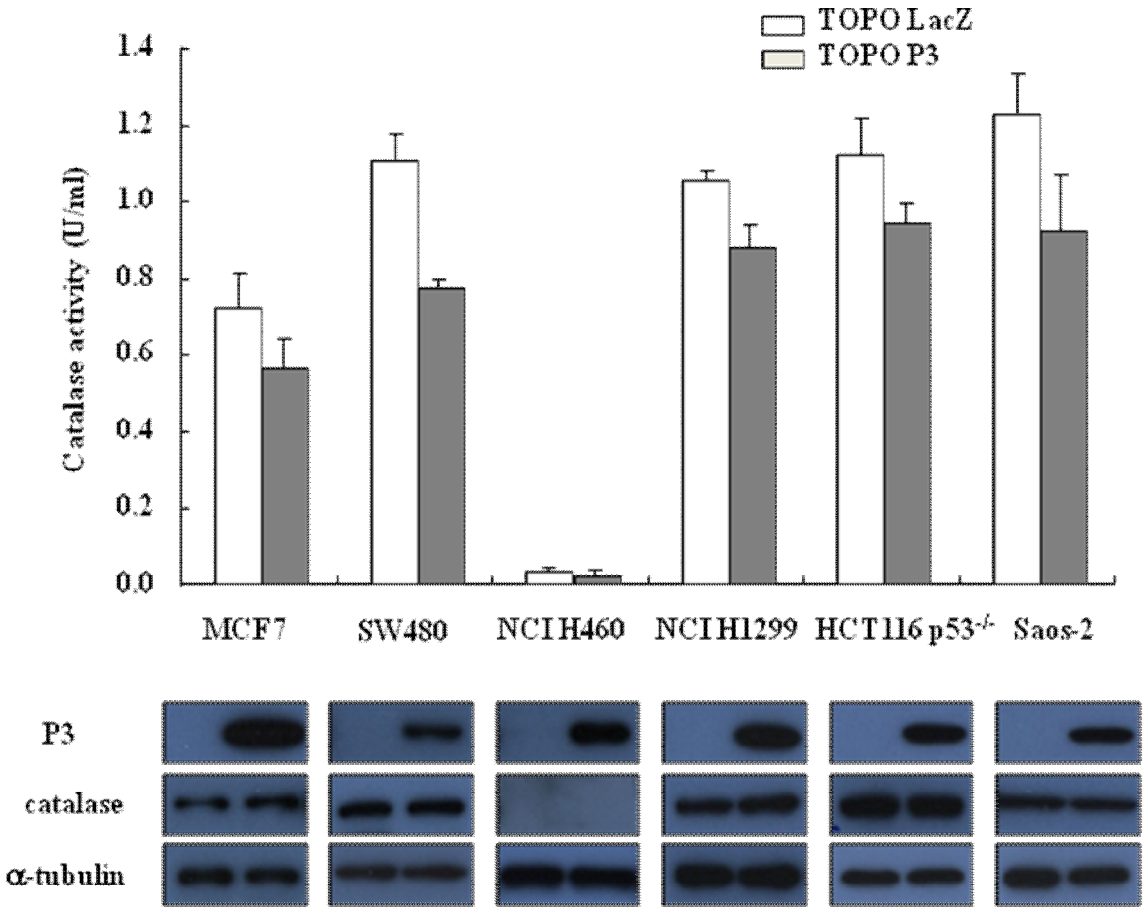

Fig.5. The effect of P3 on catalase activity *in-vivo*

A. The effect of P3 on catalase activity in different cell lines.

B. The expression patterns of P3 and catalase in difference cell lines.

We also checked ROS level after transfection of P3 into MCF7, SW480, NCI H460, NCI H1299, HCT116p53$^{-/-}$ and SaoS-2 cell lines using flow cytometry. The cell was analyzed after 48h post-transfection with control or P3 expression vectors. Results showed that the ROS level in MCF7, SW480, NCI H1299, HCT116p53$^{-/-}$ and SaoS-2 were increased about 20% after over expression of P3 but slightly decreased in NCI H460 cell line (Fig.6.).

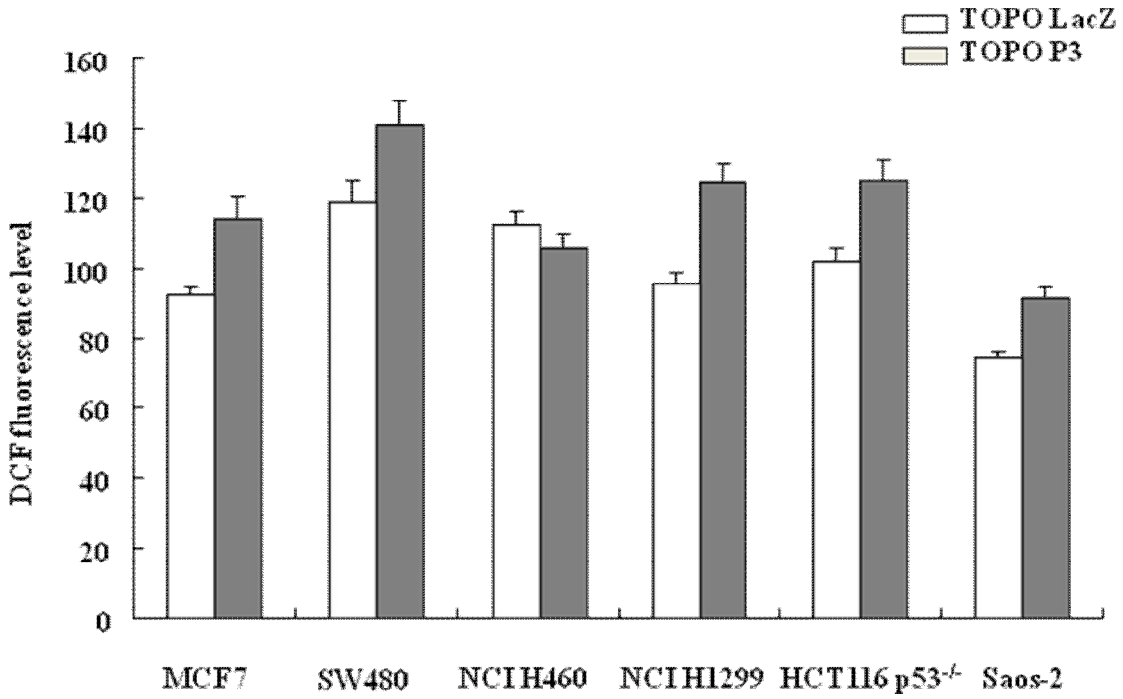

Fig.6. ROS level after transfection with P3 expression vector in different cell lines

Next, what made the difference of NCI H460 cell line in catalase activity and ROS level? After over-expresseion of P3, we compared the ROS levels in NCI H460, which were followed by infection of Adenoviral vectors, Ad GFP and Ad Catalase. In the cells which were transfected with control vector (TOPO LacZ), ROS levels were decreased after Ad Catalase infection. But P3 over-expressed cell which was followed by Ad Catalase infection showed similar level of ROS in control (Figure.7.).

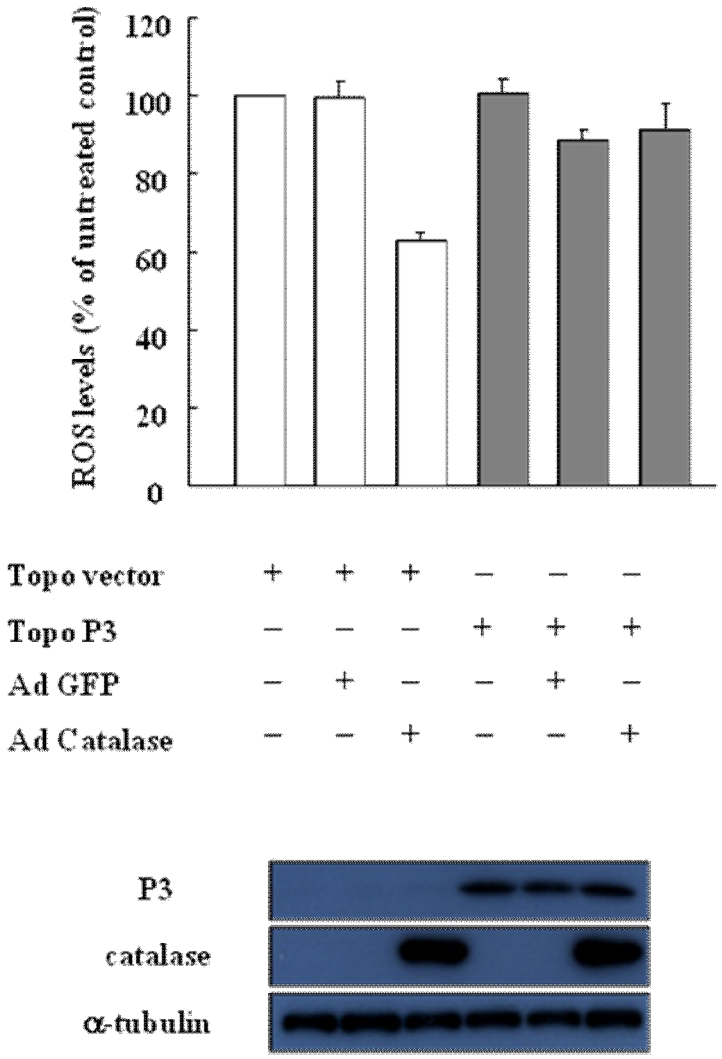

Fig.7. The ROS levels in NCI H460 cell line after Ad Catalase infection

3. Role of catalase in P3-induced apoptosis

To evaluate whether P3 protects cells from apoptosis induced by DNA damaging agents, we stably over-expressed P3 in HCT116p53$^{-/-}$ cell line. The lacZ was also transfected as a control. Then, these cells were stimulated by UV irradiation. After 48h, the catalase activity was checked using Amplex Red Catalase Assay Kit, ROS level was checked using flow cytometry and apoptotic cells were analyzed by PI staining.

In HCT116p53$^{-/-}$ (P3) cell line, ROS levels were increased about 50% after UV irradiation compared with HCT116p53$^{-/-}$ (Mock) (Fig.8.). Catalase activity in HCT116p53$^{-/-}$ (P3) cell line was decreased significantly after UV irradiation (Fig.9.). As shown in Figure.10. apoptotic cells in HCT116p53$^{-/-}$ (P3) were increased significantly.

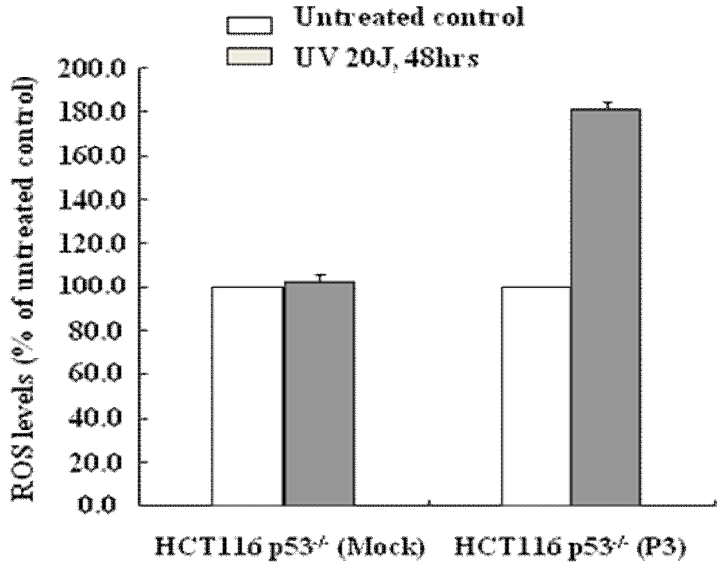

Fig.8. ROS levels were increased significantly in P3 over-expressed cell line after UV irradiation

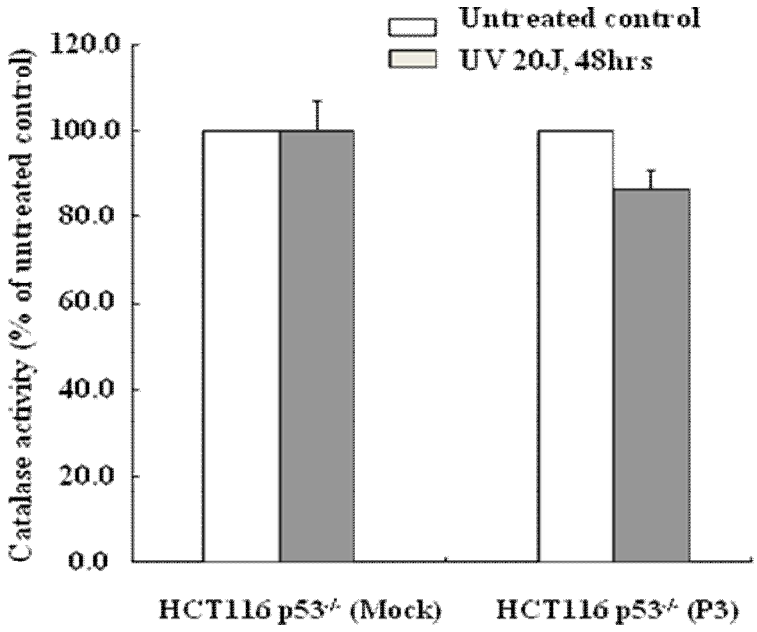

Fig.9. The catalase activity were decreased significantly in P3 over-expressed cell line after UV irradiation

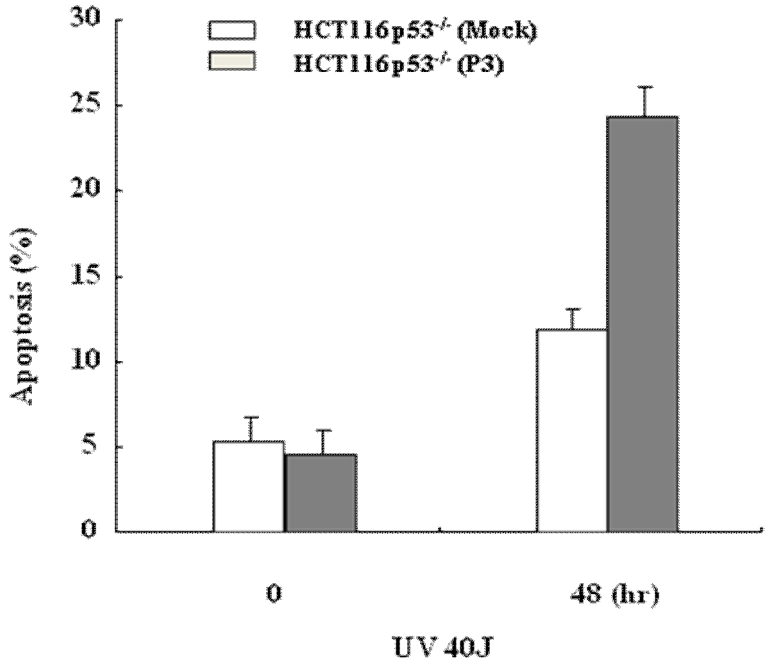

Fig.10. Apoptotic cell deaths were increased significantly in P3 over-expressed cell line after UV irradiation

IV. DISCUSSION

Several p53-target genes are involved in apoptosis according to previous studies. These genes include Bax, PIGs (p53-induced genes), Noxa, APAF-1, and PUMA. The discovery of PIGs strongly suggests that oxidative damage of mitochondria might play an important role in the p53-induced apoptosis. There is some evidence to suggest that the P3 protein is involved in the generation of ROS which are important downstream mediator of the p53-dependent apoptotic response. However, because P3 expression alone is insufficient to cause apoptosis, it is suspected that many factors cooperate to cause ROS-dependent cell death. At this time, the exact role of P3 in p53-dependent apoptosis remains to be unclear.

What is the role of P3 in inducing apoptosis? To answer this, we study a novel mechanism of P3-mediated ROS regulation. According to our results, catalase protein represents the important role in this mechanism by direct binding with P3. Through this binding, it affects intracellular ROS levels and apoptosis.

To investigate the mechanism of P3-mediated ROS regulation, we first found catalase which acted as important regulatior of ROS. We checked binding using co-immunoprecipitation, catalase activity, western blot, ROS levels using flow cytometry and PI staining for apoptosis levels. The results in Figure 2, 3 showed that P3 and catalase bind each other. Previously study showed that inhibited catalase expression has a protective role against UVB irradiation by preventing DNA damage mediated by the late ROS increase. UVB-induced apoptosis is a highly complex process involving the extrinsic and intrinsic pathways. UVB is known to be an inducer of ROS formation leading to apoptosis. It has been shown that UVB can induce the production of superoxide anion radical and hydrogen peroxide (H_2O_2). We also observed the difference of catalase activity and ROS level in some cell lines after over-expression of P3. It's clear that a difference was shown in catalase abundant and deficient cell line (Figure 5). In catalse abundant cell lines, MCF7, SW480, NCI H1299, HCT116p53$^{-/-}$ and Saos-2, the catalase activity was decreased and the ROS level was increased after over-expression P3 and we also could see it in inverse way expression in catalase deficient NCI H460. In this cell line we could check the presence of P3 and catalase at the same time related to the decrease of ROS level (Figure 7). All these results strongly imply that the binding of P3 and catalse induces the elevation of intracellular ROS importantly in a given cell line.

Next what is the role of catalase in P3-induced apoptosis? To answer this question, P3

stably over-expressed cells were made. Our data showed that the ROS level and apoptosis were increased in HCT116p53$^{-/-}$ (P3) under UV irradiation after 48h compared with HCT116p53$^{-/-}$ (Mock). During the same condition, the catalase activity was decreased significantly in P3 over-expressed cell line. All these results strongly imply that the P3 induced elevation of ROS and decreased catalase activity, importantly influence on apoptosis in a given cell.

Base on our present finding, the important role of catalase in the mechanism of P3-mediated ROS regulation was established when the catalase activity were decreased by P3 over-expression. Under that condition, ROS levels also were increased, it led to apoptosis consequently. This is the mechanism of P3-mediated ROS level regulation.

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<국문초록>

P3 에 의한 활성산소 조절기작

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종양 억제 단백질 p53 은 다양한 형태의 스트레스에 의해 활성화 되며, 세포주기 조절, DNA 복구 및 세포자살과정에 관여한다. 잘 알려져 있는 p53 의 유전적 변이는 주로 암에서 발견된다. 증가된 활성산소는 이러한 암을 유발하는 주요인자로 알려져 왔다. 몇몇 유전자는 세포내 활성산소의 수준을 조절한다고 추정되는데 그중 특히 P3 은 그 프로모터 부분에 p53 결합부위를 갖고 있으며 여러종에서 분리된 oxidoreductase 와 중요한 유사부위를 갖는다. 미토콘드리아에서 호흡 경로에 의한 산소는 superoxide 로 전환 되고, 미토콘드리아의 superoxide dismutase 에 의해 H_2O_2 가 되면서 감소된다. Catalase 는 세포내 H_2O_2 수준을 조절하는 주요 효소이며, peroxisomes 에서 H_2O_2 는 H_2O 로 전환되고 O_2 가 남는다.

본 연구에서는 활성산소에 의한 세포자살에 있어서 P3 과 catalase 의 상호관계를 규명하고자 하였다. P3 과 catalase 두 단백질의 상호결합을 규명하고자 Co-immunoprecipitation 을 실시하였고, 그 결과 in vitro 및 in vivo 상태에서 직접적인 결합을 확인할 수 있었다. 또한 U2OS 세포주에서 UV 조사후 co-localization 이 증가하는 것을 확인하였다. MCF7, SW480, NCI H460, NCI H1299, HCT116$p53^{-/-}$ 와 Saos-2 세포주에 P3 을 과발현시킨후 catalase 의 활성과 활성산소의 변화를 관찰하였다. 그결과 P3 의 과발현이 catalase 의 활성을 억제하고 따라서 활성산소의 수준도 증가시킴을 관찰하였다. 그러나 이중 NCI H460

세포주는 상이한 결과를 나타내었는데, 이는 catalase 의 발현양과 관련이 있는 것으로 보인다. 이를 증명하기 위해 P3 의 과발현후 Ad Catalase 를 감염시켜 확인한 결과 P3 에 의한 활성산소의 증가는 catalase 단백질이 발현되는 상태에서 나타났다. P3 에 의한 세포자살과정에서 catalase 의 역할을 규명하기 위해서 P3 을 과발현시킨 HCT116p53$^{-/-}$ 에 UV 조사후 실험을 진행하였다. P3 이 과발현된 세포는 UV 조사후 catalase 활성의 감소 및 활성산소의 증가 그리고 자살세포의 증가를 나타내었다.

이상의 연구결과 P3 은 활성산소에 의한 세포자살과정에 중요한 역할을 하며, 이는 catalase 의 활성과 관련이 있다. 즉 증가된 P3 의 발현은 catalase 의 활성을 억제시키며 이는 활성산소의 증가를 유도, 세포자살에 이르게 하는 것이다.

저작물 이용 허락서

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본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

2010년 10 월 14일

저작자: Nguyen Thi Kim Oanh (서명 또는 인)

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