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Sodium Salicylate Induces the Cyclin-dependent Kinase Inhibitor p21 (Waf1/Cip1) through PI3K-related Protein Kinase-dependent p53 Activation in A549 Cells

Min Young Kim, Cho Hee Kim, Jee Won Hwang, Ji Hye Kim, Hye Gyeong Park and Ho Sung Kang†

Department of Molecular Biology, College of Natural Sciences, Pusan National University, Pusan 609-735, Korea

Sodium salicylate (NaSal), a chemopreventive drug, has been shown to induce apoptosis and cell cycle arrest depending on its concentrations in a variety of cancer cells. In A549 cells, low concentration of NaSal (5~10 mM) induces cell cycle arrest, whereas it induces apoptosis at higher concentration of 20 mM. In the present study, we examined the molecular mechanism for NaSal-induced cell cycle arrest. NaSal induced expression of p53, p21 (Waf1/Cip1), and p27 (Kip1) that play important roles in cell cycle arrest. p53 induction was mediated by its phosphorylation at Ser-15 that could be prevented by the PI3K-related kinase (ATM, ATR and DNA-PK) inhibitors including wortmannin, caffeine and LY294002. In addition, NaSal-induction of p21 (Waf1/Cip1) was detected in p53 (+/+) wild type A549 cells but not in p53 (-/-) mutant H1299 cells, indicating p53-dependent p21 (Waf1/Cip1) induction. In contrast, p27 (Kip1) that is a negative regulator of cell cycle with p21 (Waf1/Cip1) was observed both in A549 cells and H1299 cells. Thus, 5 mM NaSal appeared to cause cell cycle arrest through inducing the cyclin-dependent kinase inhibitor p21 (Waf1/Cip1) via PI3K-related protein kinase-dependent p53 activation as well as by up-regulating p27 (Kip1) independently of p53 in A549 cells.

Key Words: Sodium salicylate, p21, p27, p53, PI3K-related kinase

INTRODUCTION

Sodium salicylate (NaSal) is one of non-steroidal anti-inflammatory drugs (NSAIDs) that exert its potent anti-inflammatory and antipyretic activities through their ability to inhibit cyclooxygenase activity, the key enzyme in prostaglandin biosynthesis (Tegeder et al., 2001; Amann et al., 2002). However, several clinical observations and epidemiological and experimental studies found NaSal to be a promising chemopreventive drug. Prolonged use of NaSal has been reported to reduce the risk of cancer of the colon and other gastrointestinal organs as well as cancer of the breast, prostrate, lung, and skin (Amann et al., 2002; Elder et al., 1996). The chemopreventive properties of NaSal are the result of its ability to induce apoptosis, cell cycle arrest, and differentiation in cancer cells. Several mechanisms have been proposed for NaSal-induced apoptosis, including activation of caspases, inactivation of NF-κB, and induction of several proapoptotic proteins (Elder et al., 1996; Bellosillo et al., 1999; Klampfer et al., 1999; Chan et al., 1998; Schwenger et al., 1997; Smith et al., 2000; Dikshit et al., 2006; Chung et al., 2003; Lee et al., 2003). In addition, NaSal-induced inhibition of cell proliferation in various cancer cell lines is considered to be another important mechanism for its anti-tumor activity and prevention of carcinogenesis (Shiff et al., 1996; Marra et al., 2000; Law et al., 2000). Inhibition of cell proliferation by sodium Salicylate has been shown to be mediated by up-regulation of p21 (Waf1/Cip1) and p27 (Kip1) (Marra et al., 2000). The expression of p21 (Waf1/Cip1) is usually regulated at the transcriptional level by a number of transcription factors

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†Corresponding author: Ho Sung Kang, Department of Molecular Biology, College of Natural Sciences, Pusan National University, Pusan 609-735, Korea.
Tel: 82-51-510-2275, Fax: 82-51-513-9258
e-mail: hspkang@pusan.ac.kr

including p53 and STAT (Signal Transducers and Activators of Transcription protein). The p21 promoter is also activated in a p53-independent manner upon exposure to TGF-β and IFN-γ. However, the molecular mechanism for NaSal-induced p21 (Waf1/Cip1) and p27 (Kip1) expression is not yet understood.

The tumor suppressor p53 is implicated in apoptosis or cell cycle arrest in response to a variety of genotoxic stressful stimuli (Sharpless et al., 2002; Oren, 2003; Manfredi, 2003; Ko and Preves, 1996). The tumor suppressive activity of p53 is thought to be mediated by its role as a transcription factor modulating gene expression as well as through non-transcriptional regulation at the mitochondria. Under unstressed conditions, the p53 level is very low in cells due to binding to murine double minute 2 (MDM2), a negative regulatory partner, and proteasomal degradation. Upon DNA damage by ionizing radiation or gentoxic stresses, p53-MDM2 binding is dissociated as a result of p53 phosphorylation by the PI3K-related kinases including ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR) or other kinases and acetylation by p300/pCAF, leading to p53 activation (Hammond et al., 2004; Roos and Kaina, 2006; Hurley et al., 2007). Both the N and C terminus contain sites that can be modified by phosphorylation, acetylation, and ubiquitination. Activated p53 transactivates cell cycle regulatory genes such as p21 (Waf1/Cip1) to induce growth arrest or pro-apoptotic molecules such as PUMA and BAX to induce apoptosis (Sharpless et al., 2002; Oren, 2003; Manfredi, 2003; Ko and Preves, 1996). Of critical importance to the function of p53 is the ability of tetrameric p53 to bind in a sequence specific manner to canonical sequences in the promoter or introns of response genes. The p53 DNA binding sequence, 5'PuPuPuC(A/T)(A/T)GPyPyPy3', consists of two palindromic 10 bp sequences separated by 0~13 bp.

In this study, we examined the molecular mechanism for NaSal-induced cell cycle arrest in A549 cells derived from human non-small-cell lung cancer (NSCLC) that is one of the two major categories of lung cancer that is a major cause of cancer deaths throughout the world (Takahashi, 2002). Here we show that NaSal induces cell cycle arrest through inducing p53, p21 (Waf1/Cip1), and p27 (Kip1) that play important roles in cell cycle arrest. Furthermore, we demonstrate that the cyclin-dependent kinase inhibitor p21 (Waf1/Cip1) is regulated by a PI3K-related protein kinase-dependent p53 activation, whereas p27 (Kip1) induction is regulated independently of p53 in A549 cells.

MATERIALS AND METHODS

1. Cell culture and drug treatment

Human lung adenocarcinoma cells A549 and H1299 were obtained American Type Culture Collection (ATCC) and were grown in RPMI 1640 medium (Gibco) supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) penicillin-streptomycin in a 37℃ humidified incubator with 5% CO_2. NaSal (S-3007, Sigma) was dissolved in D.W at a concentration of 1 M. For NaSal treatment, cells were incubated in the fresh media containing NaSal at different concentrations for 48 h as described in Figure legends. For the studies concerning the effects of inhibitors, A549 cells were pretreated with LY294002 (10 μM), wortmannin (0.2 μM and 20 μM), caffeine (3 mM), PD98059 (30 μM), SB203580 (10 μM), SP600125 (20 μM and 50 μM), H89, Ro-31-8220 (1 μM), or cycloheximide (Sigma, 10 μg/ml) for 1 h, followed by treatment with NaSal in the presence of the inhibitors.

2. Immunoblotting

Cells were prepared by washing with cold-phosphate-buffered saline (PBS) and lysed by adding lysis buffer (0.1% SDS, 1.0% Triton X-100 and 1.0% deoxycholate in PBS containing 1 mM DTT, 1% protease inhibitor cocktail (Sigma), 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF and 1 mM β-glycerophosphate). After incubation on ice for 30 min, the resulting extracts were centrifuged at 13,000 rpm for 20 min. The protein concentration was determined using the Bio-Rad protein assay kit. Equal amount of proteins was loaded, and separated by SDS-PAGE and then transferred to nitrocelluloses membranes. The membranes were blocked for 1 h with blocking buffer (5% skim milk, and 0.1% Tween 20 in 1 × PBS) at room temperature on a shaker. The membrane were then incubated for overnight at 4℃ with primary antibody (in 1 × PBS with 0.1% Tween 20) including antibodies to p53, phospho-p53 (Ser-15), phopho-p53 (Ser-46), p21, and p27, PARP, procaspase-3, HSP72, and ERK2 (Cell Signaling Technology). Nitrocellulose membranes were washed three times in wash buffer (1 × PBS with 0.1% Tween 20). Primary antibody was detected using 1:1,000 diluted HRP-conjugated goat anti-

mouse or goat anti-rabbit IgG antibodies and visualized with the enhanced chemiluminescence detection system (Amersham-Pharmacia Biotech, Buckinghamshire, England). Immunoblot experiments were performed at least three times.

3. Hoechst 33342 (HO)/propidium Iodide (PI)

Cells were incubated with 1 μg/ml HO and 5 μg/ml PI at 37℃, 5% CO_2 for 15 min in the dark. The cells were immediately fixed in 3.7% formaldehyde, washed with phosphate-buffered saline (PBS). The slides were washed in PBS to remove excessive dye, air dried, mounted in Fluro-Gard Antifade, and then examined by fluorescence microscopy (340/425 nm (HO), and 580/630 nm (PI) (Carl Zeiss, Axioskop 2 plus, AxioCam Mrm) as described previously (Kim et al., 2007).

4. Analysis of cell cycle arrest by FACS

Exponentially growing cells were treated with NaSal, and then collected and fixed with chilled 70% EtOH. Ten thousand stained cells with propidium iodide (PI) were analyzed on a fluorescence-activated cell sorter (FACStar-PLUS, Becton-Dicknson, San Jose, CA, USA), and the resulting DNA histogram were converted to proportions of each cell cycle phase by the ModiFit LT software (Becton-Dicknson).

5. Preparation of nuclear extracts

Cells were washed in cold PBS and rapidly frozen at -80℃. After thawing on ice, the cells were suspended first in buffer A (10 mM Tris-HCl pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT) and then in modified buffer A (10 mM Tris-HCl pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.1% NP-40). Nuclei were collected by centrifugation at 5,000 rpm for 10 min and incubated in buffer C (20 mM Tris-HCl pH 7.9, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF, 25% glycerol, 420 mM NaCl) for 15 min at 4℃. The nuclear extracts were obtained by centrifugation at 10,000 rpm for 10 min at 4℃ and stored at -80℃ for further experiments.

6. Electromobility shift assay and electromobility super-shift assay

Electromobility shift assay (EMSA) was performed according to the method described previously (Han et al., 2001). Double stranded oligonucleotides containing the p53 consensus DNA binding sequence ('5-GGGCATGTCCGG-GCATGTCC-3') or p53 mutant sequence ('5-GGGAAT-TTCCGGGAATTTCC-3') were labeled with [α-^{32}P]-ATP (Amersham, 3,000 Ci/mmol, 10 mCi/ml) by T4 oligonucleotide kinase (Promega). 15 μg of nuclear proteins was pre-incubated for 15 min at 4℃ in 19 μl of a mixture containing binding buffer (20 mM Tris-HCl pH 7.5, 5% glycerol, 40 mM NaCl, 4 mM $MgSO_4$, 1 mM EDTA, 1 mM DTT, 50 μg/ml BSA), 1 μg of poly dI-dC and 1 μl of protease inhibitor cocktail (Sigma). Binding reaction was performed for 40 min at room temperature with 1 μl (> 50,000 cpm) of radio-labeled oligonucleotide in a final volume of 20 μl. Nucleoprotein-oligonucleotide complexes were resolved by electrophoresis on a 4% acrylamide (acrylamide/bisacrylamide, 29:1) gel at 30 mA for 1 h. After electrophoresis, the gel was dried and exposed to an X-ray film for 12~24 h at -80℃. Electromobility supershift assay (EMSSA) was performed in a similar fashion to EMSA. Nuclear extracts were incubated with 2 μl of anti-p53 antibody (NeoMarkers) for 20 min at room temperature before addition of the reaction mixture containing the radioisotope-labeled p53 consensus DNA binding sequence.

7. Cell transfection and promoter deletion analysis

The human wild-type p21 promoter luciferase fusion plasmid, pWWP-Luc, was made from a 2.4-kb genomic fragment of p21 promoter containing the transcriptional start site and two p53 binding sites and then subcloned into the luciferase reporter vector, pGL-3Basic. A549 cells transfected with the vector and then treated with NaSal and then harvested to analyze the relative luciferase activity.

RESULTS AND DISCUSSION

1. Cell cycle arrest and p53-dependent p21 expression and p53-independent p27 expression by sodium salicylate

As demonstrated previously (Bellosillo et al., 1999; Klampfer et al., 1999; Chan et al., 1998; Schwenger et al., 1997; Smith et al., 2000; Dikshit et al., 2006; Chung et al., 2003; Lee et al., 2003), higher concentration of NaSal (20 mM) induced apoptosis in A549 human lung adenocarcinoma cells as determined by FACS analysis, HO/PI staining, and Western analysis for procaspase-3 activation

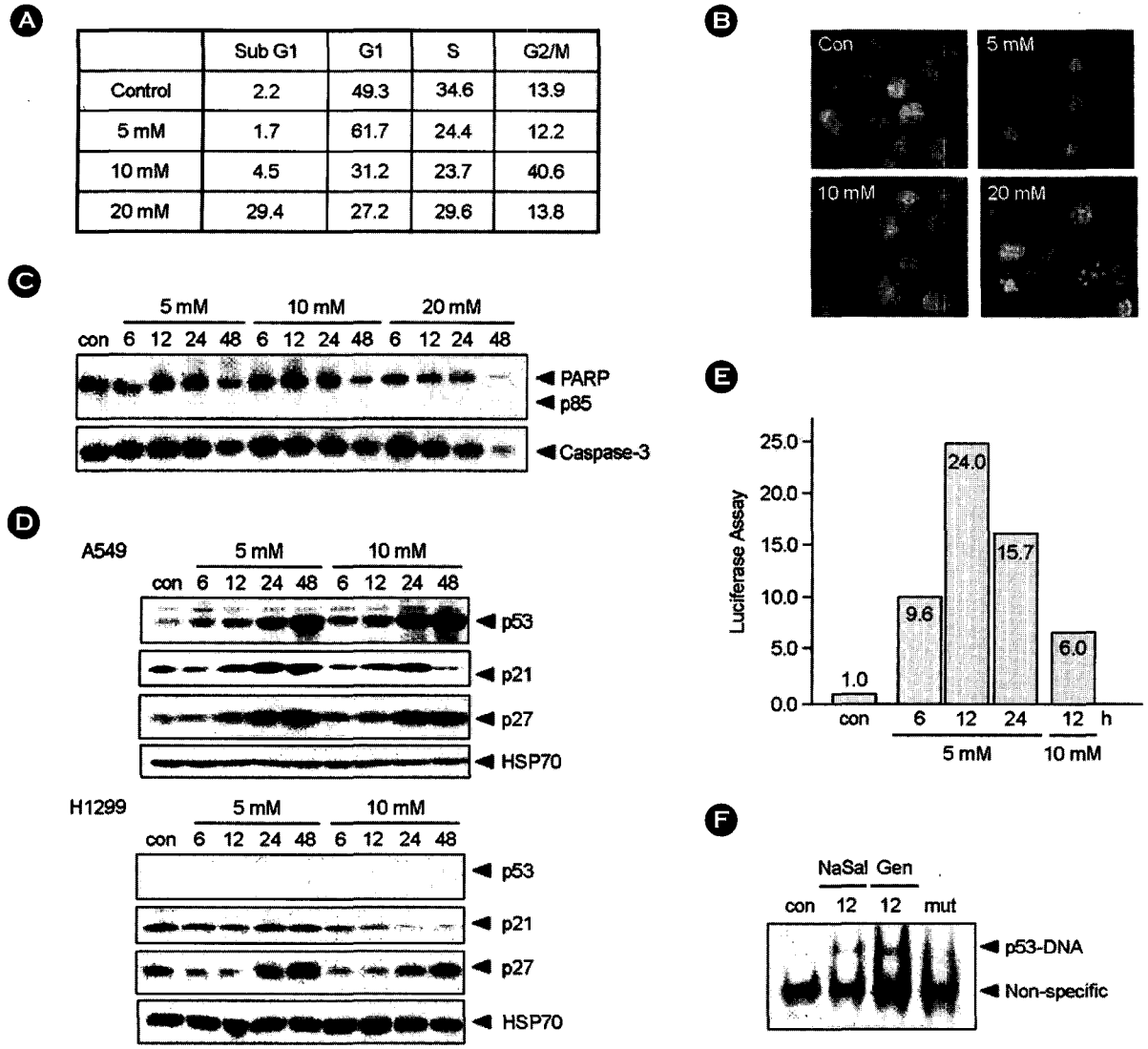

Fig. 1. Cell cycle arrest, p53-dependent p21 and p53-independent p27 expresssions by sodium salicylate. **A-B.** A549 cells were treated with 5~20 mM NaSal for 24 h and cell cycle progression and apoptotic cell death was analyzed by FACScan (A) and HO/PI staining (B), respectively. **C-D.** A549 cells were exposed to 5~20 mM NaSal for the indicated times and the resulting cell lysates were analyzed by SDS-PAGE and Western blotting with antibodies to PARP, procaspase-3, p53, p21, p27, and HSP72. **E.** p21 promoter was transfected into A549 and the cells were then treated with NaSal at the indicated concentrations for 6-12 h. The luciferase activity was normalized for the amount of protein in the cell lysate. **F.** A549 cells were exposed to 5 mM NaSal or genistein for 12 h. The nuclear proteins were then analyzed by EMSA for the p53-DNA binding. As a negative control, p53 mutant sequence ('5-GGGAATTTCCGGGAATTTCC-3') was used (mut).

and PARP cleavage (Fig. 1A-C). In contrast, lower concentration of NaSal (5~10 mM) induced cell cycle arrest (mostly G1 and G2/M arrest) without apoptotic features such as PARP cleavage (Fig. 1A and C). We observed that NaSal increased p53, a key mediator of cell cycle arrest and apoptosis, at concentrations of 5~20 mM (Fig. 1C and Fig. 2). NaSal (5~10 mM, but not at higher concentration (20 mM)) also induced p21 (Waf1/Cip1) expression (Fig. 1C). In addition, p21 (Waf1/Cip1) induction was more prominent in A549 cells treated with 5 mM NaSal than those treated with 10 mM NaSal (Fig. 1D). In addition, p21 (Waf1/Cip1) level was decreased 48 h after treatment of 10 mM NaSal. p21 promoter assay revealed that 5 mM NaSal activated p21 promoter, indicating that p21 (Waf1/Cip1) induction is regulated at the transcriptional level by 5 mM NaSal (Fig. 1D). There are two p53 binding sites in the p21 promoter, GAACA (-2234 to -2230 relative to transcriptional start site, first binding site) and AGACT (-1344 to -1340 relative to transcriptional start site, second binding site). We investigated whether p53 is responsible for NaSal

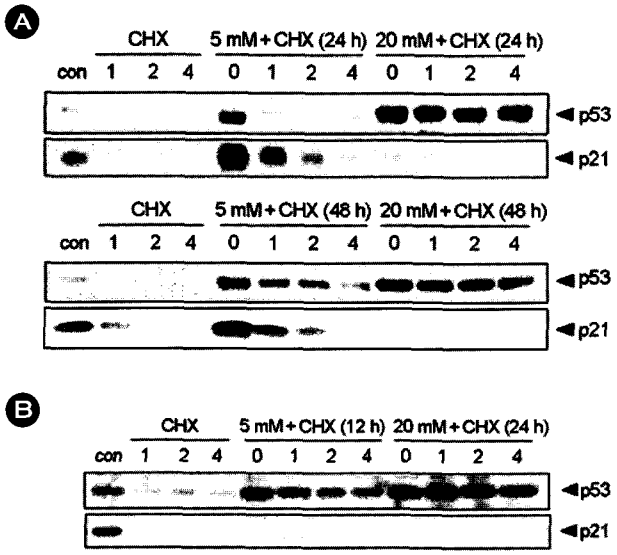

Fig. 2. Increase in p53 protein stability by treatment of sodium salicylate. **A-B**, A549 cells were exposed to 5~20 mM NaSal for the indicated times and then treated with cycloheximide (CHX) and p53 and p21 protein levels were measured by Western blot.

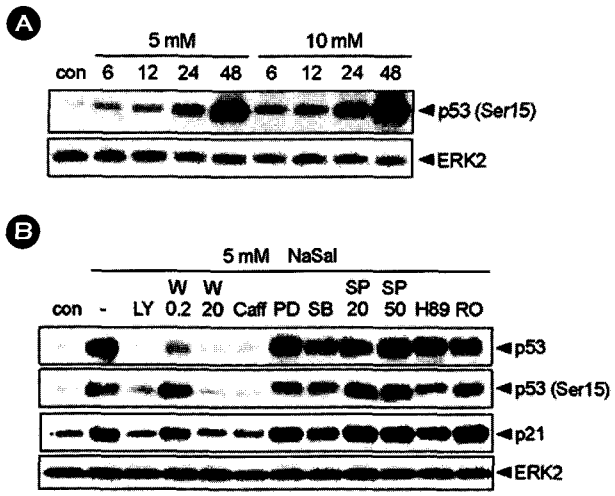

Fig. 3. PI3K-related protein kinase-dependent p53 induction and p21 expression by sodium salicylate. **A.** A549 cells were exposed to 5~10 mM NaSal for the indicated times and p53 phosphorylation at Ser 15 was examined. ERK2 was used a loading control. **B.** A549 cells were pretreated with either LY294002 (LY, 10 μM), wortmannin (W, 0.2 μM and 20 μM), caffeine (Caff, 3 mM), PD-98059 (PD, 30 μM), SB203580 (SB, 10 μM), SP600125 (SP 20 μM and 50 μM), H89, or Ro-31-8220 (RO, 1 μM) for 1 h and exposed to 5 mM NaSal for 12 h. The cellular proteins were analyzed by immunoblotting with antibodies to p53, phospho-p53 (Ser 15), p21 and ERK2.

induction of p21 (Waf1/Cip1). First, to determine whether p53 was transcriptionally active, we performed gel mobility shift assays. EMSA analysis showed that p53 binds to its responsible DNA element in response to 5 mM NaSal, indicating that p53 is transcriptionally active (Fig. 1F). In this experiment, genistein was used a positive control to induce p53 DNA-binding activity. The DNA-binding activity of p53 was peaked after 24 h upon exposure to NaSal. In addition, NaSal-induced p21 (Waf1/Cip1) expression was detected in A549 cells (wild-type p53) but not in H1299 [p53 (-/-) mutant], indicating p53-dependent p21 (Waf1/Cip1) expression (Fig. 1). In contrast, a similar induction in the level of p27 (Kip1) that is a negative regulator of cell cycle with p21 (Waf1/Cip1), was observed both in A549 cells and H1299 cells, indicating p53-independent p27 (Kip1) expression. p27 (Kip1) induction was not reduced by high dose of NaSal (20 mM).

2. PI3K-related protein kinase-dependent p53 induction and p21 expression by sodium salicylate

In normal cells, p53 is a tightly regulated protein that is maintained in low levels due to its short half-life through MDM2 binding and subsequent proteasome degradation (Sharpless et al., 2002; Oren, 2003; Manfredi, 2003; Ko and Preves, 1996). Upon DNA damage by ionizing radiation or genotoxic stresses, p53-MDM2 binding is dissociated as a result of p53 phosphorylation by PI3K-related kinases such as ATM and acetylation by p300/pCAF, leading to p53 activation (Hammond et al., 2004; Roos and Kaina, 2006; Hurley et al., 2007). To investigate the mechanism of p53 protein induction by NaSal, we compared the half-life of p53 protein in 5 mM NaSal- and 20 mM NaSal-treated cells. Cells were incubated for different time (12~48 h) in the presence of NaSal, and then cycloheximide (CHX), an inhibitor of protein synthesis, was added to inhibit further protein synthesis and p53 and p21 (Waf1/Cip1) protein levels were measured by Western blotting (Fig. 2). The half-life of p53 protein was found to be increased by treatment of NaSal. Interestingly, p53 was more stable in A549 cells treated with 20 mM NaSal compared with those treated with 5 mM NaSal. Although p53 is more stabilized and protected from proteasomal degradation, it did not induce p21 (Waf1/Cip1). These results suggest that p53 activity to cell cycle arrest may be switched to its pro-apoptotic activity as the concentration of NaSal is increased.

Since p53 is known to be phosphorylated at multiple sites and the phosphorylation leads to a stabilization of p53 by reducing its interaction with MDM2, a negative regulatory partner (Sharpless et al., 2002; Oren, 2003; Manfredi, 2003;

Ko and Preves, 1996), we investigated the changes in phosphorylation of p53 by NaSal treatment. NaSal induced phosphorylation of p53 at Ser-15, but not at Ser-46 (known as critical site for apoptosis), Ser-6, Ser-9, Ser-20, and Ser-37. p53 Ser15 phosphorylation is controlled mainly by PI3-K related kinases such as ATM, ATR, and DNA-PK in response to genotoxic stresses, and by p38MAPK in response to heat shock and cadmium chloride. Thus, we investigated which of these protein kinases is involved in Ser15 phosphorylation of p53. As shown in Fig. 3, p53 phosphorylation at Ser 15 in response to sodium salicylate was markedly prevented by wortmannin (20 μM, potent ATM and DNA-PK inhibitors), caffeine (10 mM, ATR inhibitors), LY294002 (10 μM), but not by the p38MAPK inhibitor SB203580 and the MEK1/2 inhibitor PD98059, the PKA inhibitor H89, and the PKC inhibitor Ro-31-8220, indicating that PI3K-related protein kinases is responsible for p53 phosphorylation at Ser 15 in response to NaSal.

Collectively, these results demonstrate that NaSal induces cell cycle arrest through inducing p53, p21 (Waf1/Cip1), and p27 (Kip1) that play important roles in cell cycle arrest. Furthermore, we demonstrate that the cyclin-dependent kinase inhibitor p21 (Waf1/Cip1) is regulated by a PI3K-related protein kinase-dependent p53 activation, whereas p27 (Kip1) induction is regulated independently of p53 in A549 cells. The PI3K-related kinases, ATM and ATR, are stress-response kinases and respond to a variety of insults including ionizing radiation, replication arrest, and ultraviolet radiation and activate a complex signaling network in response to diverse forms of DNA damage (Hammond et al., 2004; Roos and Kaina, 2006; Hurley et al., 2007). DNA double-strand breaks (DSBs) are detected by ATM and ATR proteins, which signal downstream to CHK1, CHK2 (checkpoint kinases) and p53. p53 induces transcriptional activation of pro-apoptotic factors such as FAS, PUMA and BAX (Hammond et al., 2004; Roos and Kaina, 2006; Hurley et al., 2007). We suspect that NaSal differs from most other stresses in that it does not induce DNA damage. Thus, NaSal may use a unique way to activate PI3K-related kinases such as ATR to induce p53-p21 (Waf1/Cip1) cascade.

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