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Naphthoquinone Analog-induced G1 Arrest is Mediated by cdc25A Inhibition and p53-independent p21 Induction in Human Hepatocarcinoma Cells

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Abstract: The naphthoquinone analog (2,3-dichloro-6,9-dihydroxy-1,4-naphtoquinone, NA) has an inhibitory effect on cdc25A protein phosphatase *in vitro*, which is responsible for G1/S transition during cell cycle. However, the exact mechanism inducing the growth inhibition is not understood. In this study, we investigated the regulatory mechanisms of growth arrest induced by NA, as a new potent inhibitor of cdc25A phosphatase, in human hepatocarcinoma SK-hep-1 cells. We found that NA induced the G1 arrest by perturbation of protein tyrosine dephosphorylation of Cdk2, which may be resulting from inhibition of cdc25A phosphatase. In addition, p21 was expressed in a p53-independent manner and participated in the NA-induced G1 arrest by inhibiting Cdk2 activity. Although the exact mechanism is not known, the p21 expression might be related to MAPK activation. From these results, we suggest that NA induces G1 arrest via inhibition of cdc25A and induction of p53-independent p21 expression in SK-Hep-1 cells.

Key words: Naphthoquinone analog (NA), G1 arrest, p53-independent p21, MAPK

Menadione (vitamin K3) is known to have growth inhibitory effects in several cell types both *in vitro* (Oztopcu et al., 2004; Markovits et al., 1998) and *in vivo* (Nishikawa et al., 1995). It has increasingly been of interest because it was shown to exhibit a broad range of antitumor activity in human cells and imposed lower level of toxicity than other cancer chemotherapeutic drugs of quinone structure, such as doxorubicin (Dunkern et al., 1999) and mitomycin C (Vallee et al., 2003; Verweij et al., 1999). However, the mechanisms involved in growth inhibition induced by menadione are still not well understood. It was previously reported that menadione inhibits the proliferation of HepG2 cells via a S/G2 arrest and altered phosphorylation patterns and activities of both p34cdc2 kinase and protein-tyrosine phosphatase (Juan et al., 1993). And also, it has been shown that a thioether analog of menadione inhibits cell growth and exerts its effect mainly via sulfhydryl arylation of cellular PTPases and redox Cycling (Brown et al., 1991; Nutter et al., 1992).

Recently, we have synthesized several vitamin K analogs and found that 2,3-dichloro-6,9-dihydroxy-1,4-naphtoquinone (NA, naphthoquinone analog) showed more potent growth inhibitory effect than other K vitamins for SK-Hep-1 cells (Ham et al., 1998). NA acts as a most strong inhibitor of cdc25A protein phosphatase, which is a specific regulator acting at the G1/S phase. Moreover, the antiproliferative activity of NA is not antagonized by exogenous thiol and nonthiol antioxidants (Ham et al., 1998), suggesting that unlike menadione, an alternative oxidant-independent pathway mediates the NA-induced cell growth inhibition.

A known major regulator of cell cycle progression in eukaryotes is Cyclin-dependent kinases (Cdks), which are inactivated by phosphorylation at Tyr-15 by Weel (Hashimoto et al., 2003) and activated by dephosphorylation at Thr14/Tyr15 by cdc25 protein phosphatase (Coulonval et al., 2003). Three cdc25 proteins are known in human cells; cdc25A, cdc25B, and cdc25C, which function at different phases of the cell cycle regulating different Cyclin-Cdk complexes (Nilsson et al., 2000; Kawabe, 2004). Especially, deregulated expression of cdc25A and cdc25B has been

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demonstrated to play an important role in some cancer cell lines derived from head, neck, and breast cancer (Ito et al., 2004; Gasparotto et al., 1997). Therefore, it is possible that NA as an inhibitor of cdc25 protein phosphatase may be involved in the inhibition of Cdk2 activity by inhibiting Tyr-15 dephosphorylation, which results in G1 arrest.

The activity of Cdks is also positively regulated by association with Cyclins and negatively regulated by binding to protein inhibitors (Dobashi, 2005; Kim and Zhao, 2005). p21(WAF1) inhibits the Cdk activity by direct binding to the active site of Cdk-Cyclin complex, and thereby leading cells to G1 arrest (Kim and Zhao, 2005; Sherr et al., 1995). In most cases, p21 expression is regulated by p53 in response to DNA damaging agents (O'Connor, 1997; Wang et al., 2003); however, p53-independent expression of p21 is also demonstrated to play the same role in G1 arrest (Zhang et al., 1995). We showed that C6-ceramide induced p53-independent p21 expression during G1 arrest and apoptosis (Kang et al., 1999; Kim et al., 2000; Oh et al., 1998). Nevertheless, the molecular mechanisms responsible for the p53-independent p21 expression are still obscure. Recent studies demonstrated that the activation of MAPK to cause cell cycle arrest was correlated with increased expression of p21 (Esposito et al., 2001; Sewing et al., 1997), although most of studies have shown that MEK/MAPK pathway functions to stimulate cellular proliferation (Robinson and Cobb, 1997). MAPK is activated by phosphorylation at its tyrosine and threonine residues by an upstream kinase, and is inactivated by phosphatases-mediated dephosphorylation. Once activated, these kinases are responsible for activation and phosphorylation of additional kinases as well as a battery of regulatory proteins including transcription factors required for the expression of genes involved in cell growth and/or differentiation (Chang and Karin, 2001). Since protein phosphatases play a critical role in controlling enzyme activity, it is possible that NA as a potent cdc25A phosphatase inhibitor may also be involved in the regulation of MAPK activation through certain protein phosphatase activation.

In this regard, we set out to investigate the regulatory mechanisms of growth arrest induced by NA as a new potent inactivator of cdc25 phosphatase in human hepatoma SK-hep-1 cells. We demonstrate that perturbation of protein tyrosine dephosphorylation, which may be resulted from cdc25 phosphatase inhibition, is responsible for NA-mediated G1 arrest. In addition, p53-independent p21 expression participates in the NA-induced G1 arrest and might be related to the MAPK activation.

MATERIALS AND METHODS

Cell Culture and Treatment

SK-Hep-1 and Hep3B cells were grown in RPMI 1640 medium (ICN Biomedical Inc., Costa Mesa, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen; Carlsbad, CA, USA) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin; Invitrogen) in a 5% CO_2 humidified incubator at 37°C. Cells were plated at a density of 3×10^5/ml on a culture dish. When the cells were approximately 60-70% confluent, they were washed once in PBS (pH 7.4), and then replaced with fresh medium containing 2% fetal bovine serum prior to the addition of NA (BIOMOL international, Plymouth Meeting, PA, USA).

Fluorescence-activated cell sorter (FACS) analysis

Cells (3×10^5/ml) were harvested at indicated time points and fixed in 70% ethanol. After fixation, medium was removed by centrifugation, and 200 μl of PBS was added to each sample. DNase-free RNase A (0.1 mg/ml) incubated for 30 min at room temperature was then added to the cells. Supernatants were then removed, and the cells were stained with 1 ml of propidium iodide (50 μg/ml) for 20 min. Fluorescence emitted from the propidium iodide-DNA complex in each cell nucleus was measured after laser excitation at 488 nm with FACS (Becton Dickinson, Franklin Lakes, NJ, USA).

Northern blot analysis

Total RNA was extracted from cells as described previously (Sambrook *et al.* 1989). The RNA (20 μg) was separated on 1% formaldehyde agarose gel and transferred onto Nylon membrane (Schleicher and Schuell Bioscience, Keene, NH, USA). Hybridization was carried out at 42°C for 16-24 h in 50% deionized formamide, 5 × SSC, 5 × Denhardt's solution, 0.5% SDS, 200 μg/ml salmon sperm DNA and [α-^{32}P]dCTP-labeled probe, which was labeled by random priming method according to the manufacturer's instructions (Roche Applied Science, Indianapolis, IN). The membrane was washed twice with buffer (2 × SSC and 0.1% SDS) at 42°C and exposed to Fuji-X-Ray film at −80°C.

Immunoprecipitation and Western Blot Analysis

Cells were washed in PBS and lysed in TENS buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 100 mM NaCl, 1% NP-40, and 1 mM phenylmethylsulfonyl fluoride). The protein concentration was determined by the Bradford method. 200 μg of the total protein was immunoprecipitated with rabbit polyclonal anti-Cdk2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by protein A-Sepharose. The Cdk2-immunoprecipitates were washed three times with TENS buffer and resolved with 20 μl Laemmli buffer. For western blot analysis of Cyclin E/D_1, Cdk2/4, pRb, p53, and p21 proteins, 100 μg of total cell lysates were separated by 7, 10, and 12% SDS-PAGE, and electrotransferred to PVDF transfer membrane (Schleicher

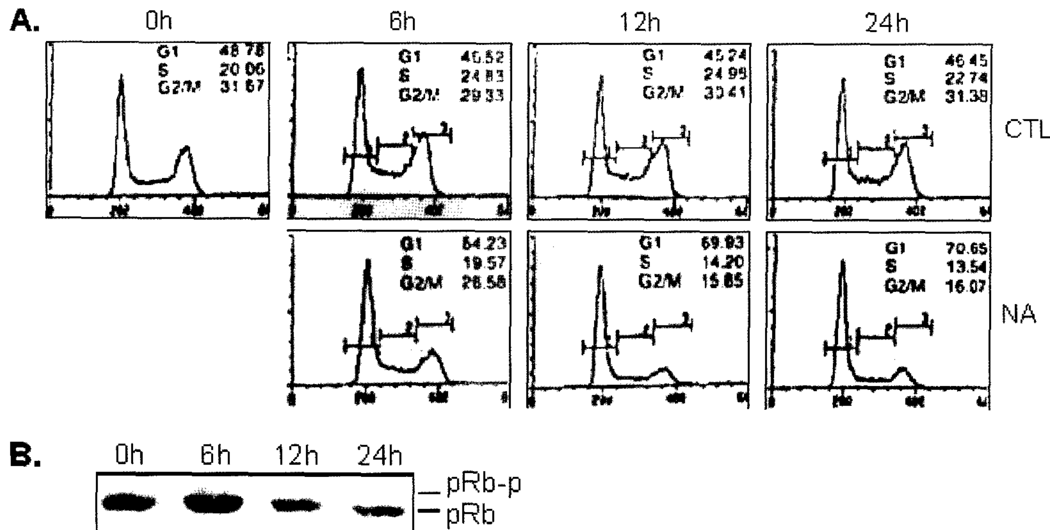

Fig. 1. NA induces cell cycle arrest and pRB dephosphorylation in SK-Hep-1 cells. (A) SK-Hep-1 cells were treated with 4 μM for the indicated periods (6, 12 and 24 h). Cells were harvested and stained with propidium iodide. Cell cycle distribution of the cells treated with or without NA was analyzed by FACS. (B) Activation of pRB was analyzed by western blot analysis employing pRB-specific antibody. pRB-p, Hyper-Phosphorylated form; pRB, Hypo-phosphorylated form.

and Schuell Bioscience). The blot was incubated with monoclonal antibody (anti-Cyclin E, -p53, and -p21) and rabbit polyclonal antibody (anti-Cdk2/4, -CyclinD1, -pRb) (Santa Cruz Biotechnology) for 15 h, followed by horseradish peroxidase-labeled secondary antibody (GE healthcare biosciences, Uppsala, Sweden) for 2 h, and then developed by enhanced-chemiluminescence (ECL) method (GE healthcare biosciences).

Histone H1 kinase assays

The Cdk2-immunoprecipitates were mixed with 20 μl of kinase buffer containing 1 mM ATP, 1.5 μCi of [γ-^{32}P] ATP, and 2 μg of histone H1. The samples were incubated at room temperature for 30 min, and the reactions were terminated by addition of 10 ml of 2 × SDS sample buffer. The samples were subjected to SDS-PAGE, and phosphorylation was detected and quantified with a densitometer.

RESULTS AND DISCUSSION

NA induces G1 arrest and pRb dephosphorylation in SK-Hep-1 cells

To initially determine whether NA blocks cell cycle progression of human hepatoma SK-Hep-1 cells, we analyzed the cell cycle distribution of NA-treated and untreated cells by FACS. NA induced G1 arrest by 24 h after the NA treatment, and G1 stage cells were gradually increased to almost 71% compared to 47% of those of untreated control cells. Since the retinoblastoma tumor suppressor protein (pRb) is known as a target molecule for G1 phase, and G1 arrest also correlates with the dephosphorylation and activation of the pRb (Kang et al., 1999), we next examined the phosphorylation status of pRb at the point when the cells underwent G1 arrest (Fig. 1B). pRb protein began to be dephosphorylated at 6 h and remained increased for up to 24 h after the NA treatment. These data demonstrated that NA induced G1 arrest of SK-Hep-1 cells in a time-dependent manner.

NA down-regulates Cyclin E-dependent kinase activity and does not alter the Cyclins/Cdks expression

It has been reported that NA potently inhibits the activity of cdc25A phosphatase *in vitro* (Ham et al., 1998). Thus, to test the possibility that NA could be involved in the inhibition of Cdk2 activity through inhibiting Tyr-15 dephosphorylation, resulting in G1 arrest of cells, we examined the NA effect on Cdk2 activation *in vivo*. Cdk2 was co-immunoprecipitated by using anti-Cyclin E antibody from cell extracts treated with or without NA at indicated time points. H1 kinase activity of Cdk2 associated with Cyclin E was reduced to about 57% by the treatment with NA for 12 h, which was significantly blocked until 24 h (Fig. 2A). To test whether the decreased Cdk2 activity was caused by dissociation of Cyclin E-Cdk2 complex, Cyclin E-immunoprecipitates were immunoblotted with Cdk2 antibodies. The level of Cdk2 associated with Cyclin E was not changed after the NA treatment, demonstrating that the inactivation of Cdk2 kinase was not due to dissociation of the Cyclin E/Cdk2 complex (Fig. 2A). In previous reports, Cdk2 activity was shown to be tightly regulated by tyrosine phosphorylation/dephosphorylation; that is, the increase in Tyr-15 phosphorylation of Cdk2 could be caused by an increase of wee1 kinase activity, or be due to a decrease of cdc25 phosphatase activity (Chen and Gardner, 2004;

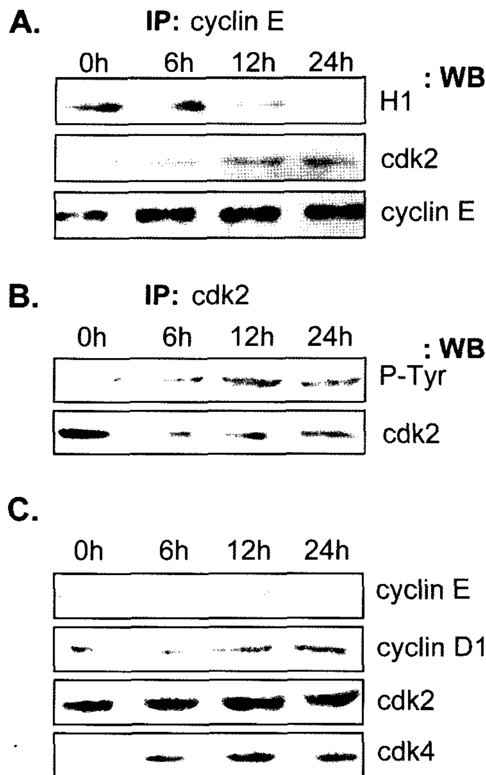

Fig. 2. The inhibition of Cdk2 activity during NA-induced G1 arrest is caused by tyrosine phosphorylation. (A) Cells treated with NA was harvested at indicated times and lysed with lysis buffer. 200 μg of total cell lysates were immunoprecipitated with antibody against Cyclin E. The immunoprecipitates were used for histone H1 kinase assay. The same immunoprecipitates were subjected to western blot analysis for Cyclin E and Cdk2. (B) 200 μg of total cell lysates were immunoprecipitated with antibody against Cdk2, and the immunoprecipitates were subjected to western blot analysis with phosphotyrosine antibody. (C) The same cell lysates were resolved by SDS-PAGE and immunoblotted with antibodies against Cyclin D1, Cyclin E, Cdk2 and Cdk4, respectively.

Kleinberger et al., 1998; Mitra and Enders, 2004). Thus, we determined whether the NA-induced inhibition of Cdk2 activity was due to negative regulatory phosphorylation of Tyr-15 *in vivo*. Cdk2 proteins were immunoprecipitated using anti-Cdk2 antibodies and then immunoblotted with anti-phosphotyrosine antibodies. As demonstrated in Fig. 2B, Cdk2 tyrosine-phosphorylation was increased from 6 h after the treatment with NA, and then increased to 3.5-fold by 24 h. These results suggested that the inactivation of Cdk2 during NA-induced G1 arrest in SK-Hep-1 cells could be due to increased Tyr-15 phosphorylation through cdc25A phosphatase inhibition.

Since relative levels of Cdks and Cyclins have also been shown to affect Cdk kinase activity in addition to the inhibition of tyrosine dephosphorylation, we investigated the relationship between the inhibitory effect of NA on the kinase activity and the change of expression levels of either Cdks themselves or their activator Cyclins. Neither Cyclin D1 nor E protein levels were affected by the NA treatment, which caused little changes in the levels of Cdk2 or Cdk4 (Fig. 2C). These results suggested that Cdk kinase inhibition was not attributed to changes of Cdks/Cyclins expression levels.

The NA induces the inhibits the p21-associated kinase

The results described above suggested that inhibition of cdc25A phosphatase, leading to an increase in tyrosine phosphorylation of Cdk2, might play an important role in the NA-induced G1 arrest. Nevertheless, the possibility that other mechanisms may also be involved in the regulation of NA-induced G1 arrest could not be excluded. p21, a well known kinase inhibitor, has been known to play a crucial role in G1 arrest by inhibiting Cyclin E/Cdk2 activity in response to several DNA damaging agents (Gasparotto et al., 1997). Thus, in order to investigate whether NA might induce p21 gene expression during the NA-mediated G1 arrest, we first examined the levels of p21 mRNA and protein after the exposure to NA in SK-Hep-1 cells. The expression of p21 mRNA and protein was remarkably increased from 6 to 24 h after the NA treatment (Fig. 3A, 3B). Since it has been known that p21 is regulated by transcriptional level through p53-dependent or independent pathways, we also examined p53 expression pattern. In contrast to p21, the expression of p53 mRNA and protein was not altered by the NA treatment (Fig. 3A, 3B), suggesting that p21 expression induced by the NA treatment was mediated by a p53-independent pathway during G1 arrest.

To test whether the p53-independent p21 expression induced by NA was responsible for the inhibition of Cdk2 kinase activity, we next measured p21 protein binding to Cyclin E/Cdk2 complex. Cell extracts were immunoprecipitated with anti-Cdk2 antibodies, followed by immunoblotting with anti-p21 antibodies. The relative amount of p21 protein bound to Cyclin E/Cdk2 complex was increased after the NA treatment, while NA did not influence the protein expression levels of Cyclin E and Cdk2 (Fig. 3C). These results demonstrated that the p53-independently increased p21 expression and the elevated p21 binding to Cdk2 inhibited the activity of Cdk2 kinase during the NA-induced G1 arrest in SK-Hep-1 cells. Although p53 expression level was not changed in NA-treated cells, p53 might still be involved in the induction of p21. Thus, to further confirm the p21 induction through a p53-independent pathway, we determined p21 expression induced by NA in p53-deficient human hepatocarcinoma cell line, Hep3B cells. As shown in Fig. 4, the p21 expression in Hep3B cells was induced by NA from 6 h to 24 h, demonstrating that p21 expression in hepatocarcinoma cells is also induced in a p53-independent manner.

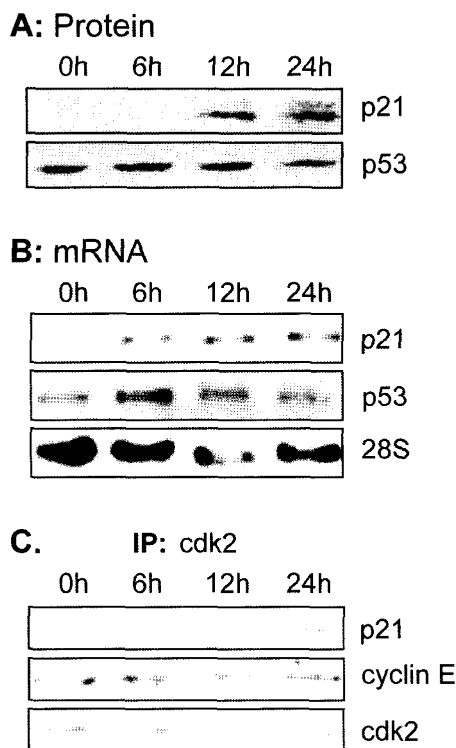

Fig. 3. p21 is induced p53-independently and associates with Cyclin E/Cdk2 complex during NA-induced G1 arrest. (A) Cells treated with NA were lysed, and 100 µg of the total cell lysates were separated by SDS-PAGE and immunoblotted with monoclonal antibodies against p53 and p21, respectively. (B), Northern blot analysis of 20 µg of the total RNA from each time point was performed as described in "Materials and Methods". As a control, the expression of 28S rRNA is also shown. (C) 200 µg of the total cell lysates were immunoprecipitated with anti-Cdk2 antibody. The immunoprecipitates were separated by SDS-PAGE and the protein level of p21 bound to Cyclin E/Cdk2 complex was analyzed by western blot analysis.

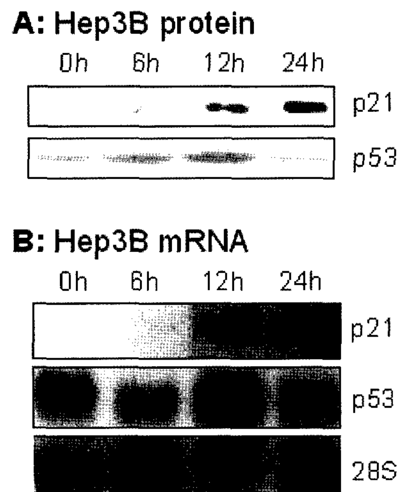

Fig. 4. p21 expression is also increased in Hep3B cells by treatment with NA. (A) Cells treated with NA were lysed, and 100 µg of the total cell lysates were separated by SDS-PAGE and immunoblotted with monoclonal antibodies against p53 and p21, respectively. (B), Northern blot analysis of 20 µg of the total RNA from each time point was performed as described in "Materials and Methods". As a control, the expression of 28S rRNA is also shown.

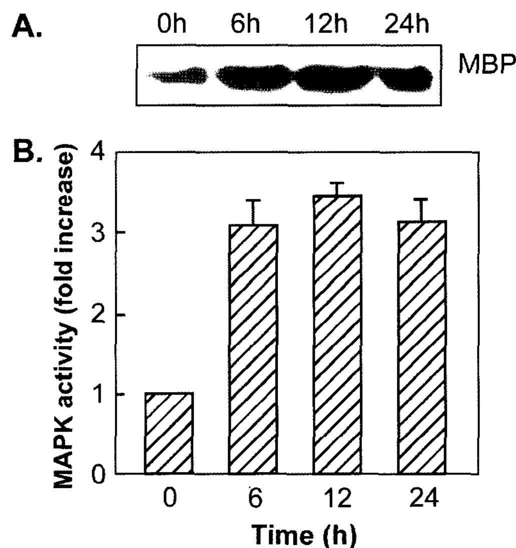

Fig. 5. MAPK is activated during NA-induced G1 arrest. (A) The MAPK activity was analyzed by a kinase assay using MBP as substrates as described in "Materials and Methods". (B) The relative activity of MAPK was quantified by densitometry analysis.

MAPK activation involves in the NA-induced G1 arrest

Since several studies have suggested that MAPK signaling pathway can induce p21 expression via p53-independent mechanisms in various cell lines, resulting in cell cycle arrest at the G1 phase, we examined the possibility that MAPK might be responsible for the induction of p21 during NA-induced G1 arrest. Treatment of SK-Hep-1 cells with NA enhanced the MAPK activity approximately 3.2 and 3.5-folds after 6 h and 12 h, respectively, which remained at about 2.8-folds above basal levels until 24 h (Fig. 5). These results were consistent with the induction time of p21 by NA, suggesting that the MAPK pathway might be involved in the induction of p21 by NA. To examine the exact role of the MEK/MAPK pathway in NA-induced G1 arrest, we next examined the effects of a specific inhibitor of MEK/MAPK, PD98059, PD98059 has shown exquisite specificity toward MEK1 relative to all other serine/threonine and tyrosine kinases assayed to date, including the closely related family members MKK3 and MKK4 (Chang and Karin, 2001). As shown in Fig. 6, the NA-induced MAPK activation was not inhibited by PD98059. Consistent with the NA-induced MAPK activity, the induction of p21 by NA was also not inhibited. In contrast, MAPK activity and p21 induced by epidermal growth factor (EGF), a Ras signaling activator, were significantly decreased. These results suggested that MAPK pathway was involved in the induction of p21 gene during the NA-induced G1 arrest, but showed that MAPK activation

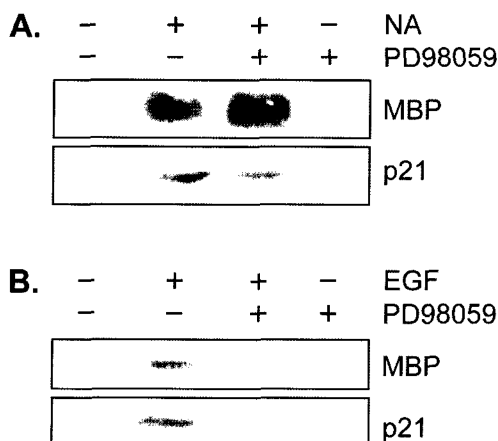

Fig. 6. The NA-induced MAPK activation is not mediated by the Ras signaling pathway. SK-Hep-1 cells were pretreated with 50 μM PD98059 for 2 h prior to treatment with NA (A) or EGF (20 nM) (B) Total cell lysates were divided into two groups. One was used for determination of MAPK using MBP, and the other for western blot analysis for p21.

by NA was mediated through a pathway different from the Ras signaling.

In summary, the present study demonstrated that the G1 arrest induced by NA was closely associated with an increase of tyrosine phosphorylation of Cdk2, which may be due to inhibition of cdc25A by NA. We found that p21 also participated in inhibition of Cdk2 by direct binding. However, p21 expression was not induced by p53-dependent pathway but may be related to MAPK in NA-induced G1 arrest in human hepatocarcinoma SK-Hep-1 cells. Further studies are required to fully determine the molecular mechanism(s) downstream of MAPK signaling that regulates p21 expression in SK-Hep-1 cells.

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REFERENCES

Brown PC, Dulik DM, and Jones TW (1991) The toxicity of menadione (2-methyl-1,4-naphthoquinone) and two thioether conjugates studied with isolated renal epithelial cells. *Arch Biochem Biophys* 285: 187-196.

Chang L, and Karin M (2001) Mammalian MAP kinase signalling cascades. *Nature* 410: 37-40.

Chen S, and Gardner DG (2004) Suppression of WEE1 and stimulation of CDC25A correlates with endothelin-dependent proliferation of rat aortic smooth muscle cells. *J Biol Chem* 279: 13755-13763.

Coulonval K, Bockstaele L, Paternot S, and Roger PP (2003) Phosphorylations of cyclin-dependent kinase 2 revisited using two-dimensional gel electrophoresis. *J Biol Chem* 278: 52052-52060.

Dobashi Y (2005) Cell cycle regulation and its aberrations in human lung carcinoma. *Pathol Int* 55: 95-105.

Dunkern TR, and Mueller KW (1999) Quantification of apoptosis induction by doxorubicin in three types of human mammary carcinoma spheroids. *Anticancer Res* 19: 3141-3146.

Esposito F, Russo L, Chirico G, Ammendola R, Russo T, and Cimino F (2001) Regulation of p21waf1/cip1 expression by intracellular redox conditions. *IUBMB Life* 52: 67-70.

Gasparotto D, Maestro R, Piccinin S, Vukosavljevic T, Barzan L, Sulfaro S, and Boiocchi M (1997) Overexpression of CDC25A and CDC25B in head and neck cancers. *Cancer Res* 57: 2366-2368.

Ham SW, Park J, Lee SJ, Kim W, Kang K, and Choi KH (1998) Naphthoquinone analogs as inactivators of cdc25 phosphatase. *Bioorg Med Chem Lett* 8: 2507-2510.

Hashimoto O, Ueno T, Kimura R, Ohtsubo M, Nakamura T, Koga H, Torimura T, Uchida S, Yamashita K, and Sata M (2003) Inhibition of proteasome-dependent degradation of Wee1 in G2-arrested Hep3B cells by TGF beta 1. *Mol Carcinog* 36: 171-182.

Ito Y, Yoshida H, Uruno T, Takamura Y, Miya A, Kuma K, and Miyauchi A (2004) Expression of cdc25A and cdc25B phosphatase in breast carcinoma. *Breast Cancer* 11: 295-300.

Juan CC, and Wu FY (1993) Vitamin K3 inhibits growth of human hepatoma HepG2 cells by decreasing activities of both p34cdc2 kinase and phosphatase. *Biochem Biophys Res Commun* 190: 907-913.

Kang KH, Kim WH, and Choi KH (1999) p21 promotes ceramide-induced apoptosis and antagonizes the antideath effect of Bcl-2 in human hepatocarcinoma cells. *Exp Cell Res* 253: 403-412.

Kawabe T (2004) G2 checkpoint abrogators as anticancer drugs. *Mol Cancer Ther* 3: 513-519.

Kim YT, and Zhao M (2005) Aberrant cell cycle regulation in cervical carcinoma. *Yonsei Med J* 46: 597-613.

Kim WH, Kang KH, Kim MY, and Choi KH (2000) Induction of p53-independent p21 during ceramide-induced G1 arrest in human hepatocarcinoma cells. *Biochem Cell Biol* 78: 127-135.

Kleinberger DN, Shelah N, Capone R, Gazit A, and Levitzki A (1998) Inhibition of Cdk2 activation by selected tyrphostins causes cell cycle arrest at late G1 and S phase. *Exp Cell Res* 241: 340-351.

Markovits J, Sun TP, Juan CC, Ju GC, and Wu FY (1998) Menadione (vitamin K3) enhances the mitogenic signal of epidermal growth factor via extracellular signal-regulated kinases. *Int J Oncol* 13: 1163-1170.

Millar JB, McGowan CH, Lenaers G, Jones R, and Russell P (1991) p80cdc25 mitotic inducer is the tyrosine phosphatase that activates p34cdc2 kinase in fission yeast. *Embo J* 10: 4301-4309.

Mitra J, and Enders GH (2004) Cyclin A/Cdk2 complexes regulate activation of Cdk1 and Cdc25 phosphatases in human cells. *Oncogene* 23: 3361-3367.

Nilsson I, and Hoffmann I (2000) Cell cycle regulation by the Cdc25 phosphatase family. *Prog Cell Cycle Res* 4: 107-114.

Nishikawa Y, Carr BI, Wang M, Kar S, Finn F, Dowd P, Zheng ZB, Kerns J, and Naganathan S (1995) Growth inhibition of hepatoma cells induced by vitamin K and its analogs. *J Biol Chem* 270: 28304-28310.

Nutter LM, Ngo EO, Fisher GR, and Gutierrez PL (1992) DNA

strand scission and free radical production in menadione-treated cells. *J Biol Chem* 267: 2474-2479.

O'Connor PM (1997) Mammalian G1 and G2 phase checkpoints. *Cancer Surv* 29: 151-182.

Oh WJ, Kim WH, Kang KH, Kim TY, Kim MY, and Choi KH (1998) Induction of p21 during ceramide-mediated apoptosis in human hepatocarcinoma cells. *Cancer Lett* 129: 215-222.

Oztopcu P, Kabadere S, Mercangoz A, and Uyar R (2004) Comparison of vitamins K1, K2 and K3 effects on growth of rat glioma and human glioblastoma multiforme cells in vitro. *Acta Neurol Belg* 104: 106-110.

Robinson MJ, and Cobb MH (1997) Mitogen-activated protein kinase pathways. *Curr Opin Cell Biol* 9: 180-186.

Sewing A, Wiseman B, Lloyd AC, and Land H (1997) High-intensity Raf signal causes cell cycle arrest mediated by p21Cip1. *Mol Cell Biol* 17: 5588-5597.

Sherr CJ, and Roberts JM (1995) Inhibitors of mammalian G1 cyclin-dependent kinases. *Genes Dev* 9: 1149-1163.

Vallee JN, Lo D, Guillevin R, Reb P, Adem C, and Chiras J (2003) In vitro study of the compatibility of tris-acryl gelatin microspheres with various chemotherapeutic agents. *J Vasc Interv Radiol* 14: 621-628.

Verweij J, Sparreboom A, and Nooter K (1999) Mitomycins. *Cancer Chemother Biol Response Modif* 18: 46-58.

Wang W, Takimoto R, Rastinejad F, and El-Deiry WS (2003) Stabilization of p53 by CP-31398 inhibits ubiquitination without altering phosphorylation at serine 15 or 20 or MDM2 binding. *Mol Cell Biol* 23: 2171-2181.

Zhang W, Grasso L, McClain CD, Gambel AM, Cha Y, Travali S, Deisseroth AB, and Mercer WE (1995) p53-independent induction of WAF1/CIP1 in human leukemia cells is correlated with growth arrest accompanying monocyte/macrophage differentiation. *Cancer Res* 55: 668-674.

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