J. Toxicol. Pub. Health
Vol. 21, No. 4, pp. 347-353 (2005)

Official Journal of
Korean Society of Toxicology
Available online at http://www.toxmut.or.kr

4-(*N*-Methyl-*N*-nitrosamino)-1(3-pyridyl)-1-butanone (NNK) Restored the Cap-dependent Protein Translation Blocked by Rapamycin

Jun-Sung Kim, Jin Hong Park, Sung-Jin Park, Hyun Woo Kim, Jin Hua, Hyun Sun Cho, Soon Kyung Hwang, Seung Hee Chang, Arash Minai Tehrani and Myung Haing Cho

Laboratory of Toxicology, College of Veterinary Medicine, and School of Agricultural Biotechnology, Seoul National University, Seoul, Korea

Received June 20, 2005; Accepted August 23, 2005

ABSTRACT. Eukaryotic initiation factor 4E (eIF4E) is a key element for cap-dependent protein translation controlled by affinity between eIF4E and 4E-binding protein 1 (4E-BP1). Rapamycin can also affect protein translation by regulating 4E-BP1 phosphorylation. Tobacco-specific nitrosamine, 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is a strong lung carcinogen, but its precise lung cancer induction mechanism remains unknown. Relative roles of cap-dependent and -independent protein translation in terms of NNK-induced lung carcinogenesis were elucidated using normal human bronchial epithelial cells. NNK concentrations applied in this study did not decrease cell viability. Addition of NNK restored rapamycin-induced decrease of protein synthesis and rapamycin-induced phosphorylation of 4E-BP1, and increased expression levels of mTOR, ERK1/2, p70S6K, and Raf-1 in a concentration-dependent manner. NNK also caused perturbation of normal cell cycle progression. Taken together, NNK might cause toxicity through the combination of restoration of 4E-BP1 phosphorylation and increase of eIF4E as well as mTOR protein expression, interruption of Raf1/ERK as well as the cyclin G-associated p53 network. Our data could be applied towards elucidation of the molecular basis for lung cancer treatment.

Keywords: NNK, Rapamycin, Cap-dependent protein translation.

INTRODUCTION

Protein translation, a crucial regulatory step in the cellular synthesis of protein in eukaryotic cells, is a multiphase process comprising initiation, elongation, and termination, which, in turn, are affected and regulated by their respective distinct factors (Bhandari *et al.*, 2001). In eukaryotic cells, different modes of initiation of translation are used depending on the nature of mRNA to be translated and physiological state of the cell (Giraud *et al.*, 2001), with two most frequently used being "scanning mechanism" and "internal initiation". In scanning mechanism, initiation of translation requires the formation of a "43S complex", which binds to 5'-m^7G cap structure of mRNA and scans along 5' UTR up to the initiator AUG. Subsequently, 60S subunit attaches to this complex, and translation is initiated (Gingras *et al.*, 1999). Internal initiation, a cap-independent mechanism, was first demonstrated in picorna viruses, which lack a 5'-m^7G cap and have long-structured 5' UTRs in their RNA (Giraud *et al.*, 2001). In addition, the presence of internal ribosome entry sites (IRES) has been detected in different viruses, such as encephalomyocarditis virus, human rhinoviruses, and hepatitis A virus (Jackson *et al.*, 1995). The IRES-mediated mechanism requires secondary structures that allow ribosomes to bind directly to the initiator AUG and translation to start without prior scanning (Giraud *et al.*, 2001), and is used under conditions where cap-dependent translation is inhibited (Mitchell *et al.*, 2001). Several genes whose protein products are associated with apoptosis contain IRES (internal ribosome entry site), including XIAP (X-linked inhibitor of apoptosis) (Holcik *et al.*, 1999), DAP5 (Death-associated protein 5) (Henis-Korenblit *et al.*, 2000), and *c-myc* (Chappell *et al.*, 1997), and can, therefore, be translated in a cap-independent manner. As reported previously, 5' untrans-

Correspondence to: Myung Haing Cho, Laboratory of Toxicology, College of Veterinary Medicine, Seoul National University, Shilim-dong, Kwanak-gu, Seoul 151-742, Korea
E-mail: mchotox@snu.ac.kr

lated region of the mRNA encoding apoptotic protease-activating factor 1 (Apaf-1) also contains IRES (Coldwell *et al.*, 2000). Thus, it can be translated via both cap-dependent and -independent manners.

Rapamycin forms a complex with immunophilin protein FKBP (FK506-binding protein), which binds to FRAP, a family of kinases (Brown *et al.*, 1994). It inhibits cap-dependent, but not cap-independent translation by modifying the phosphorylation status of eIF4E-binding protein (eIF4E-BP). Therefore, selective cap-independent translation can be triggered in rapamycin-treated cells.

Tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is formed through nitrosation of [-]-1-methyl-2-[3-pyridyl]-pyrrolidine (nicotine) during maturation, air-curing, and storage of tobacco, as well as during combustion of cigarettes (Hecht *et al.*, 1989). NNK can induce lung tumors in rodents, independent of the route of administration, and has been suggested as a causative factor in human lung cancer (Hoffmann *et al.*, 1994). Recent work revealed that susceptibility to NNK tumorigenicity is increased in transgenic mice overexpressing c-myc, which can be translated in a cap-independent manner (Ehrhardt *et al.*, 2003), strongly suggesting that both cap-dependent and -independent pathways may be important in NNK-induced tumorigenesis. Therefore, this study was performed to investigate the roles of cap-dependent and -independent protein translations in NNK-induced toxicity using normal human bronchial epithelial cells.

MATERIALS AND METHODS

Cell culture

Nontumorigenic human bronchial epithelial cells (NHBE, ATCC, USA) were grown in RPMI 1640 (Sigma-Aldrich, USA) containing 5% FBS, HEPES, sodium bicarbonate, and antibiotics (5 μg/ml). Subculturing was carried out by removing the medium, adding dissociation solution (0.25% trypsin), and allowing the flask to sit at 37°C for 12 min or until the cells detached. After subculture, fresh culture medium was added, and the cells were dispensed into new culture flasks. Fluid was renewed every 2 to 3 days. Experiments were performed when cell layers reached 80 to 90% confluence. The cells were cultured in serum-free medium for 24 h. They were then incubated, with or without pre-treatment of 20 nM rapamycin for 2 h and then, further incubation for 2 h with 50, 100 or 200 μM NNK.

MTT assay

After incubation, 50 μl of MTT [3(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide; Sigma-Aldrich] in PBS was added to each well of 96 well plates (final concentration of 0.4 mg/ml), and the cultures were incubated at 37°C in 5% CO_2 for 4 h. Subsequently, the medium was carefully removed by pipetting, and formazan crystals were dissolved in 150 μl DMSO. After 10 min agitation on a shaker, the absorbance was measured using a Thermomax micro plate reader and Softmax software (Molecular devices, USA) at wavelengths of 490 for MTT assay and 620 nm for cell viability.

Measurement of *de novo* protein synthesis

NHBE cells were cultured in serum-free medium lacking methionine for 24 h. Prior to adding to the cell cultures, rapamycin at 20 mg/ml in ethanol was diluted 1 : 1,000 in the medium to a final concentration of 20 nM. NNK was used at final concentrations of 50, 100, and 200 μM after 2 h incubation with rapamycin. Cells were randomized for 24 h incubation in the presence of 10 μCi/flask of ^{35}S-methionine (specific activity of 1000 μCi/mmol: Amersham, USA). At the end of incubation, cells were washed in phosphate-buffered saline containing 10 mmol/l methionine and lysed in 0.5 mol/l NaCl. Following precipitation in an equal volume of 24% trichloroacetic acid containing 20 mmol/l methionine, the precipitates were washed in 5% trichloroacetic acid and ethanol, and radioactivity was counted using a liquid scintillation counter (Wallac, Finland).

Measurement of protein expression by Western Blot

NHBE cells were made quiescent in serum-free media for 24 h. Following incubation with 20 nM rapamycin, the cells were then incubated with 50, 100, and 200 μM of NNK for 2 h. They were washed three times with PBS buffer and harvested in 1 ml of lysis buffer containing 50 mmol/l, Tris-HCl (pH 7.5), 150 mmol/l KCl, 1 mmol/l dithiothreitol, 1 mmol/l ethyl-enediaminetetraacetic acid (EDTA), 50 mmol/l β-glycerophosphate (pH 7.5), 1 mmol/l EGTA, 50 mmol/l sodium fluoride, 0.1 mmol/l sodium orthovanadate, 1 mmol/l, PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 10 μg/ml soybean trypsin inhibitor. Cell lysates were then sonicated on ice. The extracts were added into the sample buffer. Samples were boiled for 10 min, and proteins were separated on 15% SDS-PAGE for 18 hr. The nitrocellulose membrane was rinsed twice in Tween 20-TBS (T-TBS) with 5% skim milk. Subsequently, the membranes were incubated with 1 : 2,500 dilution of primary antibody used in our experiment in T-TBS buffer for 3 h. They were then washed twice for 10 min in T-TBS buffer, incubated with 1 : 5,000 dilution of secondary antibody conjugated to

HRP (Santa Cruz, CA, USA) in T-TBS buffer for 1 h, and washed as previously mentioned. The signals were detected using the enhanced chemiluminescence detection kit (Amersham-Pharmacia, USA).

Statistical analysis

Results are expressed as mean ± standard error (SEM) values for independence experiments. Statistical analysis was performed on all groups using *t* test.

RESULTS

Cell viability test by MTT

Cell viabilities of NHBE cells treated with NNK with or without rapamycin pretreatment were measured through MTT assay. Results revealed all groups maintained over 90% cell viability (Fig. 1). Therefore, all treatment did not cause any reduction of cell viability.

NNK recovered *de novo* protein synthesis blocked by rapamycin in NHBE cells

Effects of rapamycin and NNK on the rate of incorporation of ^{35}S-translabel into trichloroacetic acid-precipitable protein were determined as indices of *de novo* protein synthesis in NHBE cells. Rapamycin incubation for 24 h significantly decreased protein synthesis compared to the untreated control cells. The reduction, however, was recovered by co-treatment with NNK in a concentration-dependent manner. This concentration-dependent increase in *de novo* protein synthesis was also observed in the group treated with NNK only (NNK group). Overall *de novo* protein synthesis in NNK group was higher than that of rapamycin-pretreated NNK (R+NNK) group (Fig. 2).

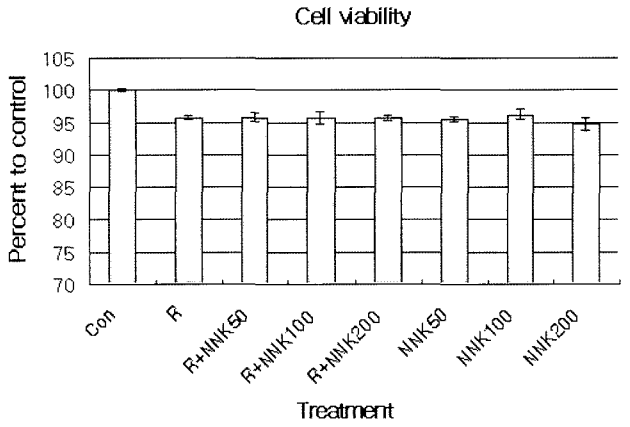

Fig. 1. Dosage response curve of NNK treatments of 50, 100, and 200 μM, and NNK treatments of 50, 100, and 200 μM with pre-treatment of rapamycin by MTT assay in NHBE cells. The NHBE cells were cultured in serum-free medium for 24 h. All experiments were processed with or without pre-treatment of 20 nM rapamycin within 2 h, followed by incubation for 2 h with 50, 100, and 200 μM NNK. Results are mean ± SEM of three experiments. R, R+50, R+100, and R+200 denote 20 nM rapamycin, and rapamycin pretreatment followed by 50, 100, and 200 μM NNK, respectively. NNK50, NNK100, and NNK200 denote 50, 100, and 200 μM NNK, respectively.

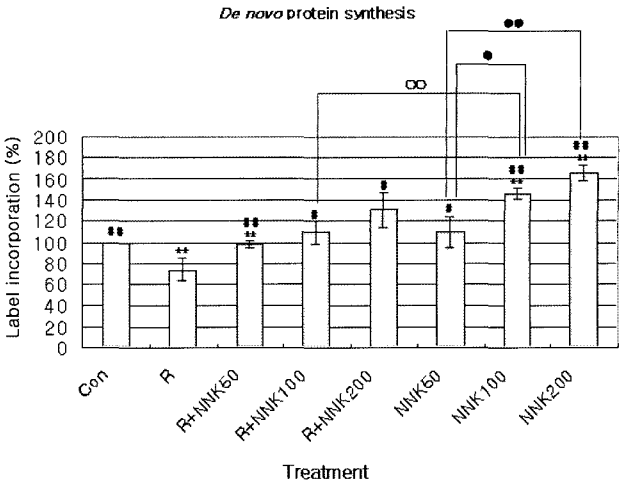

Fig. 2. *De novo* protein synthesis assay measured through the incorporation of ^{35}S- [Met] into NHBE cells. NHBE cells were cultured in serum-free medium lacking cystein and methionine for 24 h. The cells were then incubated with 10 μCi/well of ^{35}S-trans label (Amersham Biosciences, 74 MBq, 2 mCi) for 4 h in NNK with rapamycin and 2 hours with single NNK treatment. Results represent mean ± SEM of three experiments. R, R+50, R+100, and R+200 denote 20 nM rapamycin, and rapamycin pretreatment followed by 50, 100, and 200 μM NNK, respectively. NNK50, NNK100, and NNK200 denote 50, 100, and 200 μM NNK, respectively. $^{*}P < 0.05$ vs control, $^{**}P < 0.01$ vs control. $^{\#}P < 0.05$ vs rapamycin-treated group, $^{\#\#}P < 0.01$ vs rapamycin-treated group, $^{\circ}P < 0.05$ compared with each group at equal concentration of NNK between single NNK-treated groups and NNK combined with rapamycin-treated groups, $^{\circ\circ}P < 0.01$ compared with each groups in same concentration of NNK between single NNK treated groups and NNK combined with rapamycin, $^{\bullet}P < 0.05$ compared with each groups in single NNK treated groups, $^{\bullet\bullet}P < 0.01$ compared with each groups in single NNK treated groups.

NNK restored rapamycin-induced phosphorylation of 4E-BP1 in NHBE cells via changed phosphorylation of Akt and related signal pathway

NNK single treatment and co-treatment with NNK did not affect to the total Akt protein expression level, but increased the phosphorylated Akt in Threonine and Serine (Fig. 3 Akt).

The mTOR expression was increased by NNK in a concentration-dependent manner in NNK single treat-

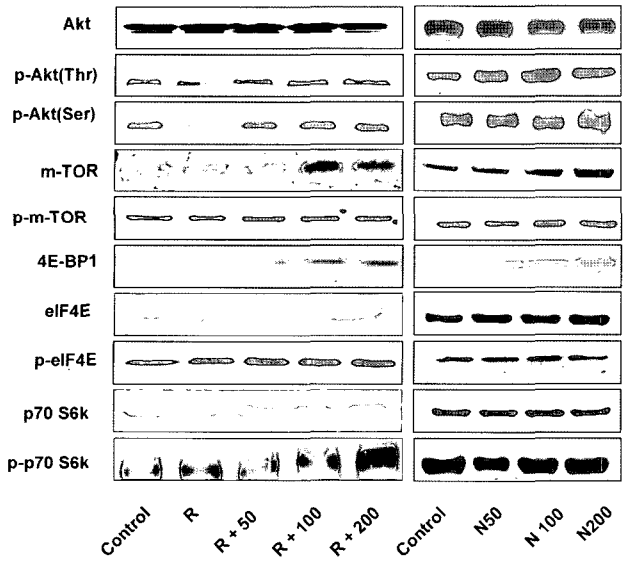

Fig. 3. Expression of Akt, p-Akt (Thr and Ser), mTOR, p-mTOR, 4E-BP1, eIF4E, p-eIF4E, p70s6k and p-p70S6K protein in NHBE cells determined by Western Blot. NHBE cells were cultured in serum-free medium for 24 h. All experiments were processed with or without of 20 nM rapamycin in 2 hours and then, incubation for 2 hours with 50, 100, and 200 μM NNK. R, R+50, R+100, and R+200 denote rapamycin of 20 nM, rapamycin pretreatment and 50, 100, and 200 μM NNK, respectively and NNK50, NNK100 and NNK200 denote 50, 100, and 200 μM NNK.

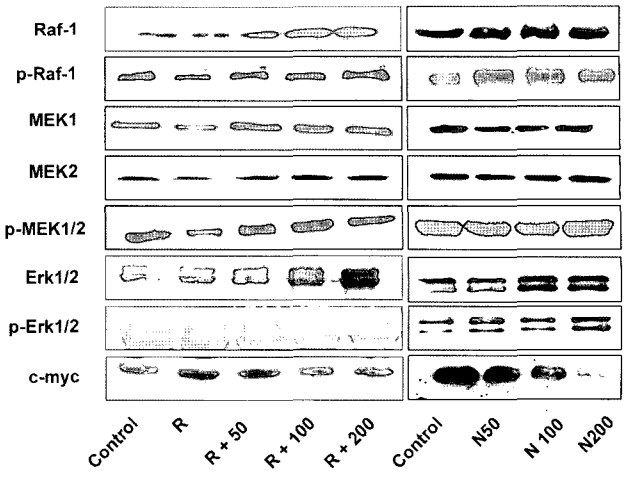

Fig. 4. Expression of Raf-1, p-Raf-1, MEK1, MEK2, pMEK1/2, Erk1/2, p-Erk1/2 and c-myc protein in NHBE cells was determined by Western Blot. NHBE cells were cultured in serum-free medium for 24 h, then all experiments were processed in with or without pre-treatment of rapamycin of 20 nM in 2 h and then, incubation for 2 h with 50, 100, and 200 μM NNK. R, R+50, R+100, and R+200 denote rapamycin of 20 nM, rapamycin pretreatment and 50, 100, and 200 μM NNK, respectively and NNK50, NNK100 and NNK200 denote 50, 100, and 200 μM NNK.

ment and co-treatment with NNK (Fig. 3 mTOR) but did not increase the phosphorylation of mTOR. In contrary, the reverse was observed in p70S6K; NNK did not cause any change in p70S6K, but, did increase the phosphorylation of p70S6K in a concentration-dependent manner. Interestingly, however, NNK group did not show such pattern (Fig. 4). Rapamycin changed the phosphorylation of the 4E-BP1, as shown through fast mobility of the protein band on Western blot. Rapamycin treatment gave not two clear different bands of eIF4E-BP1, which appeared upon co-treatment with NNK and NNK single treatment (Fig. 3 4E-BP1). Rapamycin, in addition, reduced eIF4E protein expression; however, NNK co-treatment reversed this process. NNK group also clearly showed such concentration-dependent increase of eIF4E protein expression (Fig. 3 eIF4E).

NNK increased the expression level of Raf-1 and related molecule

Co-treatment of NNK with rapamycin and single treatment of NNK increased both total Raf-1 expression and phosphorylated Raf-1 (Fig. 4 Raf-1). The similar pattern was observed in MEK1, MEK2 and their phosphorylated form. Co-treatment of NNK with rapamycin and single treatment of NNK increased Erk1/2 expression in a concentration-dependent manner, however, the increase of phosphorylated Erk1/2 was observed in NNK only group. Co-treatment of NNK did not induce any changes in the phosphorylation of Erk1/2 (Fig. 4 Erk1/2). Rapamycin increased c-myc protein expression. However, such rapamycin-induced c-myc expression was blocked by the addition of NNK in a concentration-dependent manner. Decreased c-myc protein expression was also observed in NNK group as a function of concentration (Fig. 4 c-myc).

Effect of NNK with or without rapamycin in cell cycle

Rapamycin decreased the cyclin D1 protein level, which, however, was restored by the addition of NNK. The protein expression was also induced in NNK group in a concentration-dependent manner. Similar pattern was clearly observed on cyclin E protein expression. Rapamycin increased p21 protein expression. Interestingly, R+NNK group showed decreased p21 expression in a concentration-dependent manner, whereas only high concentration of NNK showed such pattern. No significant change was observed in the p53 protein expression regardless of rapamycin pretreatment. NNK with/without rapamycin did not cause changes in the cyclin A and B protein expressions (data not shown). In

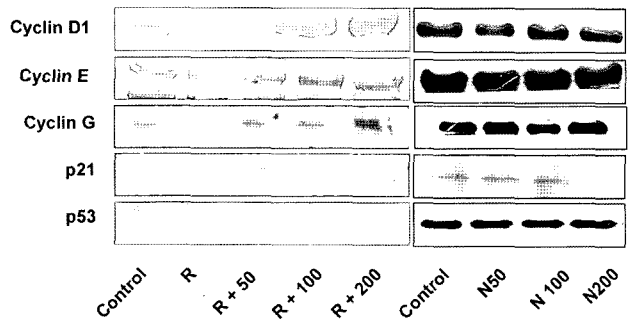

Fig. 5. Expression of cyclin D1, cyclin E, cyclin G, p21, and p53 protein in NHBE cells was determined by Western Blot. NHBE cells were cultured in serum-free medium for 24 h, then all experiments were processed in with or without pretreatment of rapamycin of 20 nM in 2 h and then, incubation for 2 h with 50, 100, and 200 μM NNK. R, R+50, R+100, and R+200 denote rapamycin of 20 nM, rapamycin pretreatment and 50, 100, and 200 μM of NNK, respectively and NNK50, NNK100 and NNK200 denote 50, 100, and 200 μM of NNK.

contrast, rapamycin decreased cyclin G protein expression significantly, although such decrease was reversed by NNK treatment in a concentration-dependent manner (Fig. 5 cyclin G).

DISCUSSION

Tobacco-specific nitrosamines are among the most important and potent carcinogenic agents in tobacco smoke. NNK, one of such nitrosamines, has been established to induce lung tumors in smokers. As observed in many other countries, the high percentage of smokers may be the main reason why lung cancer is the leading cause of cancer-related deaths in Korea. Human bronchial epithelial cell lines permanently transformed by human papilloma viruses are useful for studying growth and differentiation in bronchial carcinoma and the identification of chemical and biological agents that may be useful for the therapy of human lung cancer. Therefore, this study was performed to elucidate the precise role of NNK-induced lung tumorigenesis using normal human bronchial epithelial cells (NHBE cells).

The mammalian target of rapamycin, mTOR is a protein Ser-Thr kinase that functions as a central element in the regulation of protein translation, cell cycle progression, and cellular proliferation. Rapamycin is a highly specific inhibitor of mTOR when complexed with its cellular receptor, FKBP12, it binds directly to TOR to inhibit the downstream signaling (Fingar *et al.*, 2002). Rapamycin, thus, affect the phosphorylation of eIF4E-BP1, a key member of the eIF4E-cap complex, to induce cap-independent protein translation. Our results indicate that rapamycin affects the phosphorylation of 4E-BP1 through band shift as well as decrease of eIF4E expression (Fig. 3), which, interestingly, was reversed by NNK treatment, strongly suggesting that NNK induced toxicity through downstream cascade of mTOR signal pathway. In fact, R+NNK did not affect the expression of Akt protein, upstream of mTOR (Data not shown). Decreased *de novo* protein synthesis by rapamycin and NNK-induced concentration-dependent restoration of protein synthesis support our finding that NNK can affect mTOR-related signal cascade in a positive manner. In fact, mTOR is known to act as a sensor for mitogenic stimuli to regulate cellular growth and division. Several lines of evidences point out that tumor cells might have an advantage in growth by exploiting the rapid production of ATP to drive growth via mTOR signal pathway (Jaeschke *et al.*, 2004). Our findings in combination with those of other groups suggest that blocking of mTOR signal pathway may provide a rationale for anticipating tumor-selective suppression, because strong carcinogen NNK restores rapamycin-induced 4E-BP1 phosphorylation, and eIF4E and mTOR protein expressions. Thus, NNK might cause toxicity through the combination of restoration of 4E-BP1 phosphorylation and increase of eIF4E as well as mTOR protein expression in our system.

NNK increased ERK1/2 protein expression only in rapamycin-pretreated cells in a concentration-dependent manner. The concentration-dependent decreased effect of NNK was also observed on rapamycin-induced phosphorylation of p-Erk1/2 (Fig. 4). Our data strongly suggest that NNK affects the phosphorylation of eIF4E-BP1 and its subsequent dissociation from mRNA cap binding protein eIF4E as well as the ERK-dependent phosphorylation. Recent finding indicate that inhibition of PI3K and ERK-regulated protein syntheses using CD40, a member of TNA receptor, might be responsible for pro-apoptotic pathway (Davies *et al.*, 2003). This finding in combination with ours strongly suggests that NNK may induce toxicity through unbalanced regulation of protein synthesis machinery. In fact, we found that NNK-induced apoptosis occurred initially through cap-independent protein translation, which during later stage was replaced by cap-dependent protein translation (unpublished data). Moreover, although NNK did not induce any significant change in p70S6K, it, however, restored rapamycin-blocked phosphorylation of p70S6K in a concentration-dependent manner (Fig. 4). Dubois *et al.* (2003) reported that activation of both p70S6K and Erk was essential for T cell proliferation. Together, increased Raf-1, phospho-p70S6K as well as Erk1/2 pro-

tein may be responsible for NNK-induced toxicity in our study. Recent report revealed that inactivation of Raf/MEK/Erk was responsible for the loss of cytoprotective function in the induction of apoptosis (Yu *et al.*, 2003). Thus, interruption of Raf1/Erk pathways may be one of the underlying mechanisms of NNK-induced toxicity.

Analysis of the signaling events linking to changes in Ras/Raf/MEK/Erk cascade implicates the roles of cell cycle regulatory machinery in cell homeostasis. Erk activation was required for critical downstream effects including cyclin D1 downregulation, p21 induction, and cell cycle arrest (Clark *et al.*, 2003). NNK increased rapamycin-induced reduction of cyclin D1 expression in a concentration-dependent manner, and such concentration-dependent pattern was also observed in NNK group. Similar findings were also observed in cyclin E expression (Fig. 5). Silibinin, n active component of natural flavonoids, has been shown to have potent cancer chemopreventive efficacy. In addition, it is known to have antiproliferative and apoptotic effects through upregulation of p27 and p21 protein expressions and decreased cyclin E and D1 protein expressions (Agarwal *et al.*, 2003). These findings support our data that increase in cyclin D1 and E and decrease in p21and p53 may be responsible for NNK-induced toxicity. Interestingly, NNK also restored rapamycin-associated decrease of cyclin G protein expression in a concentration-dependent manner. Furthermore, NNK also increased cyclin G expression. Perez *et al.* (2003) reported that cyclin G overexpression was a frequent event in colorectal cancer and suggested cyclin G might be a target for gene therapy. Our data also indicate that cyclin G might be associated with downstream target of NNK, whereas cyclins A and B were not involved in NNK-induced toxicity. In fact, cyclin G is known as a key regulator of p53 network. Thus, increase of cyclin G expression may be one of the key important events for NNK-induced toxicity, because NNK decreased the corresponding p53 protein expression (Fig. 5). Cyclin G is known to form a quaternary complex *in vivo* and *in vitro* with enzymatically active phosphatase 2A holoenzymes. Therefore, cyclin G null cells have markedly higher levels of p53 protein when compared to wild-type cells. Thus, NNK might affect the cyclin G-associated p53 network in a negative manner, decreasing the expression of p50 while increasing that of cyclin G. Such discrepancy may be also one of the underlying causes of NNK-induced toxicity.

NNK is believed to be a causative agent of lung cancer in smokers. To exert its carcinogenic potential, NNK is metabolically activated by cytochrome P450-catalyzed alpha-hydroxylation (Keyler *et al.*, 2003). Some researchers reported that normal human bronchial epithelial cells may have limited ability to catalyze the metabolic activation of NNK. They used four different transfected cell lines overexpressing P450s 2A6, 3A4, 2F1, and 2E1, and found that P450 transfection fortifies 3-methyl-indole toxicity. Several lines of evidences, however, show that human lung cells contain some degree of metabolic capability. Smith *et al.* reported (2003) that peripheral human lung microsome produced total NNK bioactivation (represented by the sum of the four alpha-carbon hydroxylation endpoint metabolites) ranging from 0.002100 to 0.005685% alpha-hydroxylation/mg of protein/min. Such low amount of NNK activation was nonetheless greater than that of the detoxication products. Contributions of enzymes to NNK oxidation were CYP2A6 and/or CYP2A13, as well as CYP2B6. In addition, Agopyan *et al.* (2003) found that airborne particulate matter is activated by immortalized human bronchial epithelial cells, normal human bronchial/tracheal epithelial cells, and normal human small airway epithelial cells from the distal airways, suggesting that such cell line may have some degree of metabolic capability. Pohjola *et al.* (2003) also found that soluble extracts of particle matters produced dose-dependent benzo(*a*)pyrene-DNA adduct in the normal human bronchial epithelial cell line, indicating that the cells used in this study contain metabolic capability. As further revealed through high-density DNA microarray, smoke-induced gene expression and regulation were clearly observed in normal human epithelial cells (Yoneda *et al.*, 2003). Above results demonstrate complex genes could be expressed in normal human bronchial epithelial cells in response to the insult of environmental pollutants including smoke (Yoneda *et al.*, 2003).

In conclusion, NNK might cause toxicity through the combination of 4E-BP1 phosphorylation restoration and increase of eIF4E as well as mTOR protein expression, interruption of Raf1/ERK, and cyclin G-associated p53 network. Our data could be applicable for the elucidation of the molecular basis for lung cancer treatment.

ACKNOWLEDGEMENT

This work was supported by the Brain Korea 21 project. Kee-Ho Lee is supported by grants from the Basic Research Program of the Korea Science and Engineering Foundation (R01-2000-000-00089-0), and National R & D Program of the Korean Ministry of Science and Technology.

REFERENCES

Agarwal, C., Singh, R.P., Dhanalakshmi, S., Tyagi, A.K., Teck-

lenburg, M., Sclafani, R.A. and Agarwal, R. (2003): Silibinin upregulates the expression of cyclin-dependent kinase inhibitors and causes cell cycle arrest and apoptosis in human colon carcinoma HT-29 cells. *Oncogene*, **22**, 8271-8282.

Agopyan, N., Bhatti, T., Yu, S. and Simon, S.A. (2003): Vanilloid receptor activation by 2- and 10-microm particles induces responses leading to apoptosis in human airway epithelial cells. *Toxicol. Appl. Pharmacol.*, **192**, 21-35.

Bhandari, B.K., Feliers, D., Duraisamy, S., Stewart, J.L., Gingras, A.C., Abboud, H.E., Choudhury, G.G., Sonenberg, N. and Kasinath, B.S. (2001): Insulin regulation of protein translation repressor 4E-BP1, and eIF4E-binding protein, in renal epithelial cells. *Kidney International,* **59**, 866-875.

Brown, E.J., Albers, M.W., Shin, T.B., Ichikawa, K., Keith, C.T., Lane, W.S. and Schreiber, S.L. (1994): A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature*, **369**, 756-758.

Chappell, S.A., LeQuesne, J.P., Paulin, F.E., de Schoolmeester, M.L., Stoneley, M., Soutar, R.L., Ralston, S.H., Helfrich, M.H. and Willis, A.E. (2002): A mutation in the c-myc-IRES leads to enhanced internal ribosome entry in multiple myeloma: a novel mechanism of oncogene de-regulation. *Oncogene*, **19**, 4437-4440.

Clark, J.A., Black, A.R., Leontieva, O.V., Frey, M.R., Pysz, M.A., Kunneva, L., Woloazynska-Read, A., Roy, D. and Black, J.D. (2003): Involvement of the ERK signaling cascade in protein kinase C-mediated cell cycle arrest in intestinal epithelial cells. *J. Biol. Chem.*, in press.

Coldwell, M.J., Mitchell, S.A., Stoneley, M., MacFarlane, M. and Willis, A.E. (2000): Initiation of Apaf-1 translation by internal ribosome entry. *Oncogene*, **19**, 899-905.

Davies, C.C., Mason, J., Wakelam, M.J., Young, L.S. and Eliopoulos A.G. (2003): Inhibition of PI3K and ERK MAPK-regulated protein synthesis reveals the pro-apoptotic properties of CD40 ligation in carcinoma cells. *J. Biol. Chem.,* in press.

Dubois, S., Shou, W., Haneline, L.S., Fleisher, S., Waldmann, T.A. and Muller, J.R. (2003): Distinct pathways involving the FK506-binding proteins 12 and 12.6 underlie IL-2 versus IL-15-mediated proliferation of T cells. *Proceedings of the National Academy of Sciences*, **100**, 14169-14174.

Ehrhardt, A., Bartels, T., Klocke, R., Paul, D. and Halter, R. (2003): Increased susceptibility to the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in transgenic mice overexpressing c-myc and epidermal growth factor in alveolar type II cells. *J. Cancer Res. Clin. Oncol.*, **129**, 71-75.

Fingar, D.C., Salama, S., Tsou, C., Harlow, E. and Blenis, J. (2002): Mammalian cell size in controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E. *Genes and Development*, **16**, 1472-1487.

Gingras, A.C., Gygi, S.P., Raught, B. and Polakiewicz, R.D. (1999): Abraham RT, Hoekstra MF, Aebersold R, and Sonenberg N. Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. *Genes & Development*, 1422-1437.

Giraud, S., Greco, A., Brink, M., Diaz, J.J. and Delafontaine, P. (2001): Translation initiation of the insulin-like growth factor I receptor mRNA is mediated by an internal ribosome entry site. *J. Biol. Chem.*, **23**, **276**, 5668-5675.

Hecht, S.S., Morse, M.A., Amin, S., Stoner, G.D., Jordan, K.G., Choi, C.I. and Chung, F.L. (1989): Rapid single-dose model for lung tumor induction in A/J mice by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and the effect of diet. *Carcinogenesis*, (10), 1901-1904.

Henis-Korenblit, S., Strumpf, N.L., Goldstaub, D. and Kimchi, A. (2000): A novel form of DAP5 protein accumulates in apoptotic cells as a result of caspase cleavage and internal ribosome entry site-mediated translation. *Mol. Cell Biol.*, **20**, 496-506.

Hoffmann, D., Brunnemann, K.D., Prokopczyk, B. and Djordjevic, M.V. (1994): Tobacco-specific N-nitrosamines and Areca-derived N-nitrosamines: chemistry, biochemistry, carcinogenicity, and relevance to humans. *J. Toxicol. Environ. Health*, (1), 1-52.

Holcik, M., Sonenberg, N. and Kormeluk, R.G. (2000): Internal ribosome initiation of translation and the control of cell death. *Translation and Cell Death*, 469-473.

Jaeschke, A., Dennis, P.B. and Thomas, G. (2004): mTOR: a mediator of intracellular homeostasis. *Curr. Top. Microbiol. Immunol.*, **279**, 283-298.

Keyler, D., Pentel, P.R., Kuehl, G., Collins, G. and Murphy, S.E.: Effects of nicotine infusion on the metabolism of the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in rats. *Cancer Letter*, **202**, 1-9, 2003. Mitchell and Tollervey D. mRNA turnover. *Curr. Opin. Cell Biol.*, **13**, 320-325. 2001.

Perez, R., Wu, N., Klipfel, A.A. and Beart, R.W. Jr. (2003): A better cell cycle target for gene therapy of colorectal cancer: cyclin G. *J. Gastrointest Surg.*, **7**, 884-889.

Pohjola, S.K., Lappi, M., Honkanen, M., Rantanen, L. and Savela, K. (2003): DNA binding of polycyclic aromatic hydrocarbons in a human bronchial epithelial cell line treated with diesel and gasoline particulate extracts and benzo[*a*]pyrene. *Mutagenesis*, **18**, 429-438.

Smith, G.B., Bend, J.R., Bedard, L.L., Reid, K.R., Petsikas, D. and Massey, T.E. (2003): Biotransformation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in peripheral human lung microsomes. *Drug Metab.Dispos.*, **31**, 1134-1141.

Yoneda, K., Chang, M.M., Chmiel, K., Chen, Y. and Wu, R. (2003): Application of high-density DNA microarray to study smoke- and hydrogen peroxide-induced injury and repair in human bronchial epithelial cells. *J. Am. Soc. Nephrol.*, **14**, S284-289.

Yu, C., Subler, M., Rahmani, M., Reese, E., Krystal, G., Conrad, D., Dent, P. and Grant, S. (2003): Induction of apoptosis in BCR/ABL$^+$ cells by histone deacetylase inhibitors involves reciprocal effects on the RAF/MEK/ERK and JNK pathways. *Cancer Biol. Ther.*, **2**, 544-551.