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Cap-independent protein translation is initially responsible for 4-(*N*-methylnitrosamino)-1-(3-pyridyl)-butanone (NNK)-induced apoptosis in normal human bronchial epithelial cells

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Evidences show that eukaryotic mRNAs can perform protein translation through internal ribosome entry sites (IRES). 5'-Untranslated region of the mRNA encoding apoptotic protease-activating factor 1 (Apaf-1) contains IRES, and, thus, can be translated in a cap-independent manner. Effects of changes in protein translation pattern through rapamycin pretreatment on 4-(methylnitrosamino)-1-(3-pyridyl)-butanone(NNK, tobacco-specific lung carcinogen)-induced apoptosis in human bronchial epithelial cells were examined by caspase assay, FACS analysis, Western blotting, and transient transfection. Results showed that NNK induced apoptosis in concentration- and time-dependent manners. NNK-induced apoptosis occurred initially through cap-independent protein translation, which during later stage was replaced by cap-dependent protein translation. Our data may be applicable as the mechanical basis of lung cancer treatment.

Key words: Cap-dependent protein translation, NNK, Apoptosis

Introduction

Protein translation, an important step in the cellular protein synthesis of eukaryotic cells, is a multiphase process in which each phase, that is, initiation, elongation, and termination, is affected and regulated by distinct factors [3,7]. In eukaryotic cells, different modes of translation initiation are used depending on the nature of mRNA to be translated and physiological state of the cell [10], with two most frequently used being "scanning mechanism" and "internal initiation". In scanning mechanism, initiation of translation requires the formation of "43S complex", which binds to 5'-m^7G cap structure of mRNA and scans along 5' UTR up to the initiator AUG [21]. Subsequently, 60S subunit attaches to this complex, and translation is initiated [9]. Internal initiation, a cap-independent mechanism, was first demonstrated in picorna viruses, which lacks a 5'-m^7G cap and have long-structured 5' UTRs in their RNA [10]. In addition, the presence of internal ribosome entry sites (IRES) has been shown in different viruses, such as encephalomyocarditis virus, human rhinovirus, and hepatitis A virus. This IRES-mediated mechanism requires secondary structures that allow ribosomes to bind directly to the initiator AUG and permit translation to start without prior scanning [10], and is used under conditions where cap-dependent translation is inhibited [25]. Several genes whose protein products are associated with apoptosis contain IRES, including XIAP [16], DAP5 [14], and c-*myc* [31], and can, therefore, be translated in a cap-independent manner. As reported previously, 5' untranslated region of the mRNA encoding apoptotic protease-activating factor 1 (Apaf-1) has IRES. Thus, it can be translated via both cap-dependent and independent manners.

Apoptosis, an active as well as morphologically distinct form of programmed cell death, occurs largely under physiological conditions [19,20,22,32] with critical roles of Apaf-1 [25]. When the cells are exposed to stress and cytotoxic agent, mitochondria play a central role in the execution of apoptosis [30]. The mitochondria release cytochrome C in the presence of dATP, and form an apoptosome, which is composed of Apaf-1 and procaspase 9, resulting in caspase 9 activation. Caspase 9, in turn, activates effector procaspases such as procaspase 3, to initiate apoptosis [8,28]. Caspases are a family of cysteine proteases, which are activated during apoptosis, and play an essential role in programmed cell death process. The activation of caspase 3, in particular, is extremely important, because it is the most biologically relevant effector caspases

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identified to date, being responsible for the cleavage of a large number of target proteins [23,24,26,27].

Rapamycin forms a complex with immunophilin protein FKBP (FK506-binding protein), which binds to FRAP, a family of kinases [4]. It inhibits cap-dependent, but not cap-independent translation through modifying the phosphorylation status of eIF4E binding protein (eIF4E-BP). Therefore, selective cap-independent translation can be produced in rapamycin-treated cells [2].

Tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is formed by nitrosation of [-]-1-methyl-2-[3-pyridyl]-pyrrolidine (nicotine) during maturation, air-curing, and storage of tobacco, as well as during combustion of cigarettes [13,15,29]. NNK can induce lung tumors in rodents, independent of route of administration, and has been suggested as a causative factor in human lung cancer [15]. In this study, the relative roles of cap-dependent and/or -independent protein translations in NNK-induced apoptosis have been evaluated using human bronchial epithelial cells.

Materials and Methods

Chemicals

NNK (CAS NO. 64091-91-4) was obtained from Chemsyn Science Laboratories (Lenexa, USA), with over 99% purity as revealed through HPLC analysis (data not shown). NNK was dissolved in absolute ethanol containing 5% dimethylsulfoxide (DMSO, Sigma, USA) to form a 20 mM stock solution. For *in vitro* use, dilutions of stock solution were made in RPMI 1640 (Gibco, USA) without fetal bovine serum (FBS, Hyclone Lab, USA). Rapamycin (Sigma, USA) was reconstituted in DMSO and used at a final concentration of 20 nM.

Cell culture and treatment

Human bronchial epithelial cells (ATCC Number: CRL-2503) were cultured in RPMI 1640 supplemented with 10% (v/v) FBS, and maintained at 37°C in an atmosphere of 5% CO_2 in air. Cells were treated with 50, 100, and 200 μM of NNK for 2 hrs with/without rapamycin pretreatment. For concentration-dependent study, cells were treated with 200 mM of NNK for 4, 12, and 24 hrs with/without rapamycin.

Determination of cell viability

Cell viability of NNK with/without rapamycin on cells was determined by measuring 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma, USA) dye absorbance of living cells. One hundred microliter of the cell suspension was plated in 96-well microliter plate (Nunc, Denmark) in 2×10^5 cells/well. After incubation for 24 hrs, the cells were exposed to NNK with/without rapamycin. At the end of treatment, 10 μl of MTT solution (1 mg/ml in PBS) was added to each well, and the plates were incubated for additional 4 hrs at 37°C. After removing media, 100 μl of DMSO was added to each well. The plates were shaken for 10 min at room temperature, and the absorbance was measured at 540 nm in a microplate reader (Molecular Devices, USA).

Western blot analysis

After incubation, the cells were washed in PBS, suspended in lysis buffer [50 mM Tris at pH 8.0, 150 mM NaCl, 0.02% sodium azide, 1% sodium dodecyl sulfate (SDS), 100 μg/ml phenylmethylsulfonylfluoride, 1 μl/ml aprotinin] and centrifuged at 12,000 × *g* for 15 min. Protein concentration was determined using Bradford analysis kit (Bio-Rad, USA). Equal amounts of the protein were separated on 15% SDS gel and transferred onto nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech, USA). The blots were blocked for 2 hrs at room temperature with blocking buffer (5% nonfat milk in TTBS buffer containing 0.1% Tween 20). The membrane was incubated for 3 hrs at room temperature with specific antibodies. Then the membrane was reincubated for 1 hr at room temperature with horseradish peroxidase (HRP) conjugated secondary antibodies. β-Actin was used as an internal control. Protein bands were detected by enhanced chemilunescence (ECL, USA) detection kit (Amersham Pharmacia Biotech, USA).

Fluorometric caspase activity assay

A total of 2×10^5 cells were lysed in lysis buffer containing 25 mM HEPES (pH 7.4), 5 mM EDTA, and 2 mM DTT. The lysates were clarified by centrifugation, and supernatants were used for enzyme assays. Caspase 3 substrate (Ac-DEVD-AMC) and caspase 9 substrate (LEHD-AMC) were purchased from Calbiochem (Darmstadt, Germany). And, the specific inhibitors for caspase 3 (AC-DEVD-CHO), caspase 9 (LEHD-CHO) (Calbiochem) were used. Caspase assay was carried out using fluorogenic substrates, according to the protocol provided by the manufacturer. Reaction mixtures were incubated at 37°C for 1 hr, and fluorescence was measured using a fluorometer (Hitachi F-2000 Fluorescence Spectrophotometer, Japan) with excitation and emission at 360 and 460 nm, respectively.

Flowcytometric detection of apoptosis

Apoptosis was determined by staining the cells with annexin V for phosphatidylserine (PS) exposure and propidium iodide (PI) for cell permeability. Cells were incubated on ice with cold annexin binding buffer, PI, and annexin V according to the manufactures instructions, and were analyzed with a FACStar flowcytometer (Becton Dickinson, USA).

Transient transfection assay

Cells were cultured and transfected with bicistronic constructs (pcDNA-*f*Luc-polIRES-*r*Luc) (kindly donated by Dr. Gram, Novartis, Switzerland), and pGL3 Apaf-1 promoter

construct (kindly donated by Dr. Helin, European Institute of Oncology, Italy) using FuGene 6 transfection reagent (Roche, Germany). Transfected cells were incubated for 48 hrs in a 5% CO_2 incubator. After incubation, the cells were exposed to NNK with/without rapamycin for appropriate periods, harvested, and lysed. Cell extracts were analyzed for renilla and firefly luciferase following the suppliers instruction (Promega, USA).

Statistical analysis

Results are shown as mean ± SE. Statistical analyses were performed following analysis of variance (ANOVA) for multiple comparisons or Students *t*-test when data consisted of only two groups. Differences between groups were considered significant at $p < 0.05$ and $p < 0.01$.

Results

Determination of cell viability

Cell viabilities of NNK-treated human bronchial epithelial cells as determined by MTT assay, showed no significant differences in a concentration-response study except NNK 200 mM with rapamycin pretreatment (Fig. 1A), whereas decreased in a time-dependent study (Fig. 1B). All of control and rapamycin alone showed more than 90% cell viabilities, indicating that rapamycin itself did not cause any damage on cell viabilities. Cell viabilities maintained above 80% even at 24 hrs of NNK with/without rapamycin (Fig. 1B).

Measurement of caspase activity

Western blot analysis

a. Concentration-dependent expressions of caspase 3 and 9 protein In Western blot analysis, caspase 3 and 9 protein levels of rapamycin-treated cells increased compared with those of control. Regardless of rapamycin pretreatment, NNK increased caspase 3 and 9 protein expressions. Densitometric analysis revealed that caspase 3 and 9 protein levels of NNK alone increased in a concentration-dependent manner, whereas such concentration-dependent pattern was not observed in NNK+rapamycin group (Fig. 2A, Densitometric data not shown).

b. Time-dependent expresions of caspase 3 and 9 protein There were clear concentration-dependent increase of caspase 3 and 9 protein expressions in NNK-treated group with highest level at 24 hrs NNK. In contrast, however, rapamycin-pretreated NNK group did not have such trend. Regardless of NNK concentration with rapamycin pretreatment, both of caspase 3 and 9 expressions remained unchanged (Fig. 2B).

Fluorometric measurement for caspase activity

a. Caspase 3 and 9 activities increased in a concentration-dependent manner Caspase 3 activities showed clear concentration-dependent increases in NNK alone as well as NNK+rapamycin. In contrast, similar concentration-dependent increase of caspase 9 activities were observed in NNK , whereas such clear pattern was not found in NNK+rapamycin. Multiple comparisons showed that overall caspase 3 activities in NNK with rapamycin were significantly lower than those of NNK alone. NNK+rapamycin-induced caspase 3 activities were even lower than that of rapamycin control. Interestingly, rapamycin induced significant increase of caspase 3, whereas it did not increase caspase 9 activity. Specific

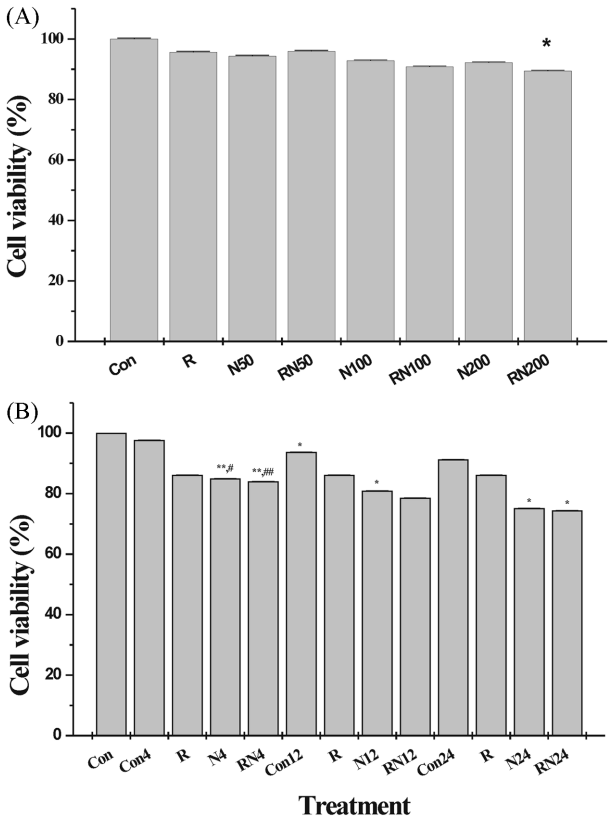

Fig. 1. Effects of NNK on cell viability of human bronchial epithelial cells. (A) After 2 hrs following NNK treatment, concentration dependency of viability was estimated by MTT assay as described in Materials and Methods. Values represent mean ± SE (n = 3). Statistical significance of difference from control (*p < 0.05). Dunnetts test for multiple comparisons and Students *t*-test. C: Control; R: Rapamycin; N50: NNK 50 μM; N100: NNK 100 μM; N200: NNK 200 μM; RN50: Rapamycin + NNK 50 μM; RN100: Rapamycin + NNK 100 μM; RN200: Rapamycin + NNK 200 μM (B) After 200 μM of NNK treatment, cell viability in a time-dependent manner was estimated by MTT assay as described in Materials and Methods. Values represent mean ± SE (n = 3). Statistical significance of difference from control (*$p < 0.05$, **$p < 0.01$) and control 4 hrs ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$). Dunnett's test for multiple comparisons and Students *t*-test. C: Control; Con4: Control 4 hrs; Con12: Control 12 hrs; Con24: Control 24 hrs; R: Rapamycin, N4: NNK, 4 hrs; RN4: Rapamycin + NNK, 4 hrs; N12: NNK, 12 hrs; RN12: Rapamycin + NNK, 12 hrs; N24: NNK, 24 hrs; RN24: Rapamycin + NNK, 24 hrs.

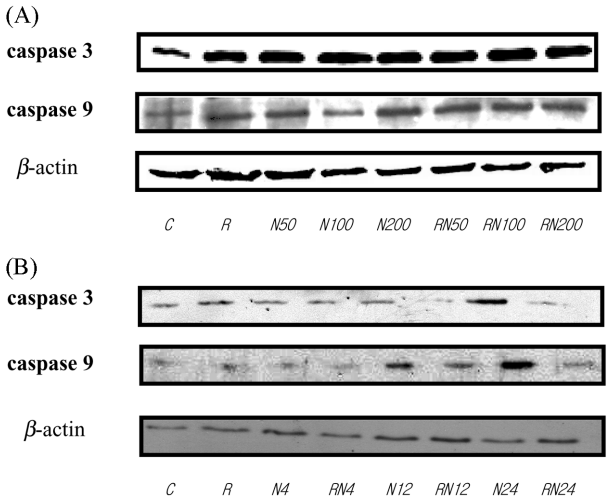

Fig. 2. (A) Concentration-dependent effects of caspase 3 and 9 protein expressions in treatment of NNK with/without rapamycin after 2 hrs following NNK treatment. Protein was prepared for Western blot analysis with appropriated primary and secondary antibodies, as described in Materials and Methods. C: Control; R: Rapamycin; N50: NNK 50 μM; N100: NNK 100 μM; N200: NNK 200 μM; RN50: Rapamycin + NNK 50 μM; RN100: Rapamycin + NNK 100 μM; RN200: Rapamycin + NNK 200 μM (B) Time-dependent effects of caspase 3 and 9 protein expressionsin treatment of NNK with/without rapamycin after 200 μM of NNK treatment. Protein was prepared for Western blot analysis with appropriated primary and secondary antibodies, as described in Materials and Methods. C: Control; R: Rapamycin; N4: NNK, 4 hrs; RN4: Rapamycin + NNK, 4 hrs; N12: NNK, 12 hrs; RN12: Rapamycin + NNK, 12 hrs; N24: NNK, 24 hrs; RN24: Rapamycin + NNK, 24 hrs.

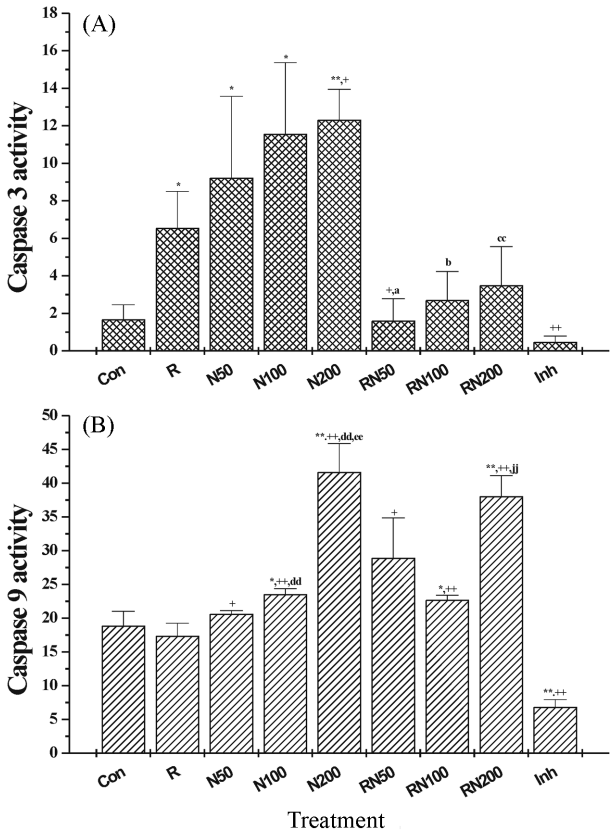

Fig. 3. (A) Effects of NNK with/without rapamycin on caspase 3 activation in concentration-dependent treatment after 2 hrs following NNK treatment. To determine the caspase activity, cell lysates were incubated with fluorogenic peptide substrates at 37°C for 60 minutes as described in Materials and Methods section. Results are means ± SE (n = 3). Statistical significance of difference from control (*$p < 0.05$, **$p < 0.01$), rapamycin ($^{+}p < 0.05$, $^{++}p < 0.01$), NNK 50 μM ($^{a}p < 0.05$), NNK 100 μM ($^{b}p < 0.05$), NNK 200 μM ($^{cc}p < 0.01$). Dunnetts test for multiple comparisons and Students *t*-test. C: Control; R: Rapamycin; N50: NNK 50 μM; N100: NNK 100 μM; N200: NNK 200 μM; RN50: Rapamycin + NNK 50 μM; RN100: Rapamycin + NNK 100 μM; RN200: Rapamycin + NNK 200 μM; Inh: inhibitor (B) Effects of NNK with/without rapamycin on caspase 9 activation in concentration-dependent treatment after 2 hrs following NNK treatment. To determine the caspase activity, cell lysates were incubated with fluorogenic peptide substrates at 37°C for 60 minutes as described in Material and Methods section. Results are means ± SE (n = 3). Statistical significance of difference from control (*$p < 0.05$, **$p < 0.01$), rapamycin ($^{+}p < 0.05$, $^{++}p < 0.01$), NNK 50 μM ($^{dd}p < 0.01$), NNK 100 μM ($^{ee}p < 0.01$), rapamycin + NNK 50 μM ($^{jj}p < 0.01$). Dunnetts test for multiple comparisons and Students *t*-test. C: Control; R: Rapamycin; N50: NNK 50 μM; N100: NNK 100 μM; N200: NNK 200 μM; RN50: Rapamycin + NNK 50 μM; RN100: Rapamycin + NNK 100 ìM; RN200**:** Rapamycin + NNK 200 ìM; Inh: inhibitor.

inhibitors for caspase 3 and 9 convinced that all experiments were performed properly (Fig. 3 A and B).

b. Time-dependent patterns of caspase 3 and 9 activities Activity of caspase 3 with NNK was higher than those of NNK with rapamycin. Interestingly, the activities of NNK with/without rapamycin decreased until 12 hrs, then, increased sharply (Fig. 4A). However, the such pattern was not reproduced in caspase 9 activity study. In fact, caspase 9 activities of NNK alone did not induce any significant changes, whereas rapamycin pretreatment group showed clear concentration-dependent increase (Fig. 4B). Also, specific inhibitors for caspase 3 and 9 inhibit corresponding caspase, respectively (Fig. 4 and B).

Western blotting of Bax, Bid, Bcl-2, and Cytochrome c

Concentration-dependent changes of Bax, Bid, Bcl-2, and Cytochrome c protein expressions

Bax, Bid, Bcl-2, and Cytochrome c protein levels were not affected by rapamycin pretreatment. Regardless of rapamycin pretreatment, NNK increased Bax, and Bid protein expression in a concentration-dependent manner. Interestingly, overall level of expression was higher in NNK+rapamycin that that of NNK alone (Fig. 5). Concentration-dependent increases of Bcl-2 and Cytochrome c protein were observed in NNK alone, while both protein levels remained unchanged in NNK+rapamycin (Fig. 6).

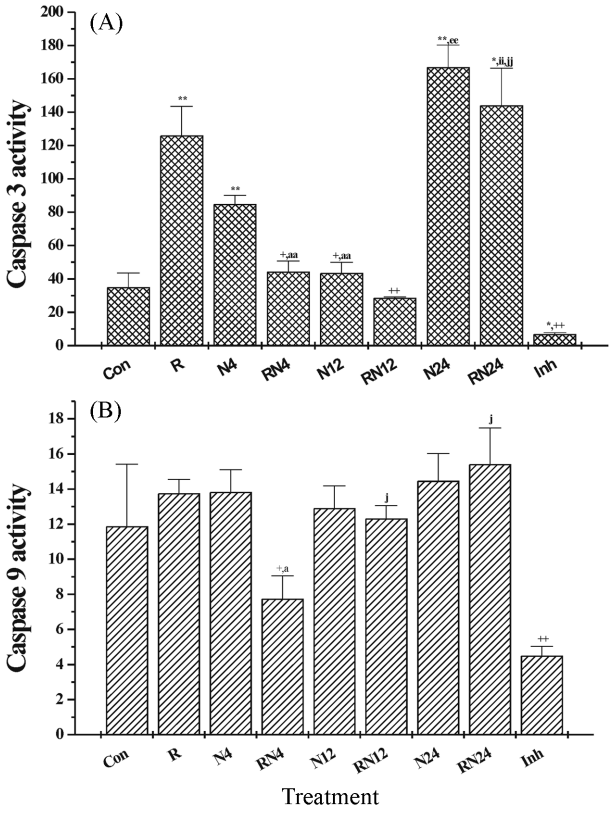

Fig. 4. (A) Time course effects of NNK with/without rapamycin on caspase 3 activation after 200 μM of NNK treatment. To determine the caspase activity, cell lysates were incubated with fluorogenic peptide substrates at 37°C for 60 minutes as described in Material and Methods section. Results are means ± SE (n = 3). Statistical significance of difference from control (*$p < 0.05$, **$p < 0.01$), rapamycin ($^{+}p < 0.05$, $^{++}p < 0.01$), NNK 4 hrs ($^{aa}p < 0.01$), NNK 12 hrs ($^{ee}p < 0.01$), rapamycin + NNK 4 hrs ($^{ii}p < 0.01$), rapamycin + NNK 12 hrs ($^{ii}p < 0.01$). Dunnetts test for multiple comparisons and Students *t*-test. C: Control; R: Rapamycin; N4: NNK, 4 hrs; RN4: Rapamycin + NNK, 4 hrs; N12: NNK, 12 hrs; RN12: Rapamycin + NNK, 12 hrs; N24: NNK, 24 hrs; RN24: Rapamycin + NNK, 24 hrs; Inh: inhibitor (B) Time course effects of NNK with/without rapamycin on caspase 9 activation after 200 μM of NNK treatment. To determine the caspase activity, cell lysates were incubated with fluorogenic peptide substrates at 37°C for 60 minutes as described in Material and Methods section. Results are mean ± SE (n = 3). Statistical significance of difference from rapamycin ($^{+}p < 0.05$, $^{++}p < 0.01$), NNK 4 hrs ($^{a}p < 0.05$), rapamycin + NNK 4 hrs ($^{j}p < 0.05$). Dunnetts test for multiple comparisons and Students *t*-test. C: Control; R: Rapamycin; N4: NNK, 4 hrs; RN4: Rapamycin + NNK, 4 hrs; N12: NNK, 12 hrs; RN12: Rapamycin + NNK, 12 hrs; N24: NNK, 24 hrs; RN24: Rapamycin + NNK, 24 hrs; Inh: inhibitor.

Time-dependent changes of Bax, Bid, Bcl-2, and Cytochrome c protein level

Rapamycin treatment induced significant increase in both Bax and Bid protein expression. NNK treatment increased Bax protein expression in time-dependent manner, however, rapamycin pretreatment did not change any protein levels of Bax as well as Bid protein expression. NNK alone did not induce any time-dependent change of Bid protein expression, either (Fig. 7). However, there was no significant changes of Bcl-2 and cytochrome c protein expression (Data not shown).

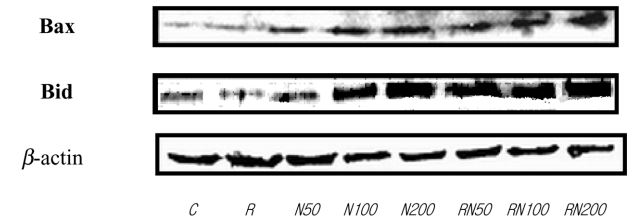

Fig. 5. Concentration-dependent effects of Bax and Bid protein expressions in treatment of NNK with/without rapamycin after 2 hrs following NNK treatment. Protein was prepared for Western blot analysis with appropriated primary and secondary antibodies, as described in Materials and Methods. C: Control; R: Rapamycin, N50: NNK 50 μM; N100: NNK 100 μM; N200: NNK 200 μM; RN50: Rapamycin + NNK 50 μM; RN100: Rapamycin + NNK 100 μM; RN200: Rapamycin + NNK 200 μM.

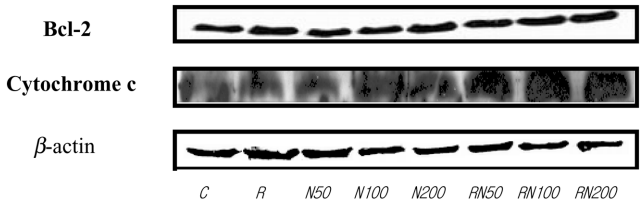

Fig. 6. Concentration-dependent effects of Bcl-2 and cytochrome c protein expressions in treatment of NNK with/without rapamycin after 2 hrs following NNK treatment. Protein was prepared for Western blot analysis with appropriated primary and secondary antibodies, as described in Materials and Methods. C: Control; R: Rapamycin; N50: NNK 50 μM; N100: NNK 100 μM; N200: NNK 200 μM; RN50: Rapamycin + NNK 50 μM; RN100: Rapamycin + NNK 100 μM; RN200: Rapamycin + NNK 200 μM.

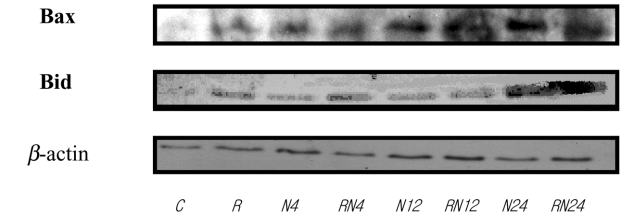

Fig. 7. Time-dependent effects of Bax and Bid protein expressions in treatment of NNK with/without rapamycin after 200 μM of NNK treatment. Protein was prepared for Western blot analysis with appropriated primary and secondary antibodies, as described in Materials and Methods C: Control; R: Rapamycin; N4: NNK, 4 hrs; RN4: Rapamycin + NNK, 4 hrs; N12: NNK, 12 hrs; RN12: Rapamycin + NNK, 12 hrs; N24: NNK, 24 hrs; RN24: Rapamycin + NNK, 24 hrs.

a. Expression of Apaf-1, eIF4E, and FRAP protein levels Apaf-1 protein was highly expressed by rapamycin treatment. NNK and NNK+rapamycin induced concentration-dependent increase in eIF4E protein expression. Whereas no

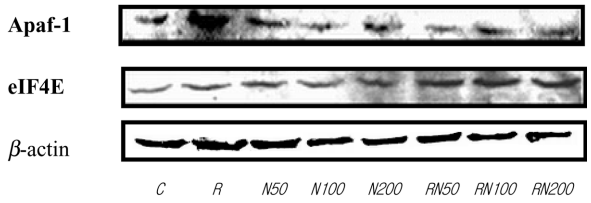

Fig. 8. Concentration-dependent effects of Apaf-1 and eIF4E protein expressions in treatment of NNK with/without rapamycin after 2 hrs following NNK treatment. Protein was prepared for Western blot analysis with appropriated primary and secondary antibodies, as described in Materials and Methods. C: Control; R: Rapamycin; N50: NNK 50 μM; N100: NNK 100 μM; N200: NNK 200 μM; RN50: Rapamycin + NNK 50 μM; RN100: Rapamycin + NNK 100 μM; RN200: Rapamycin + NNK 200 μM.

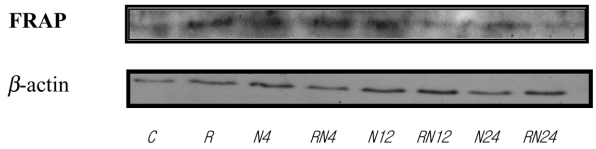

Fig. 9. Time-dependent effects of FRAP protein expressions in treatment of NNK with/without rapamycin after 200 μM of NNK treatment. Protein was prepared for Western blot analysis with appropriated primary and secondary antibodies, as described in Materials and Methods. C: Control; R: Rapamycin; N4: NNK, 4 hrs; RN4: Rapamycin + NNK, 4 hrs; N12: NNK, 12 hrs; RN12: Rapamycin + NNK, 12 hrs; N24: NNK, 24 hrs; RN24: Rapamycin + NNK, 24 hrs.

significant changes of Apaf-1 were observed in NNK wit/ without rapamycin (Fig. 8). The FRAP protein expression increased in concentration-dependent manner by both NNK and NNK+rapamycin. However, regardless of rapamycin pretreatment, such expressions decreased in time-dependent manner (Fig. 9).

b. Flow cytometric analysis of NNK-induced apoptosis

To determine the apoptosis, human bronchial epithelial cells treated with NNK with/without rapamycin were stained

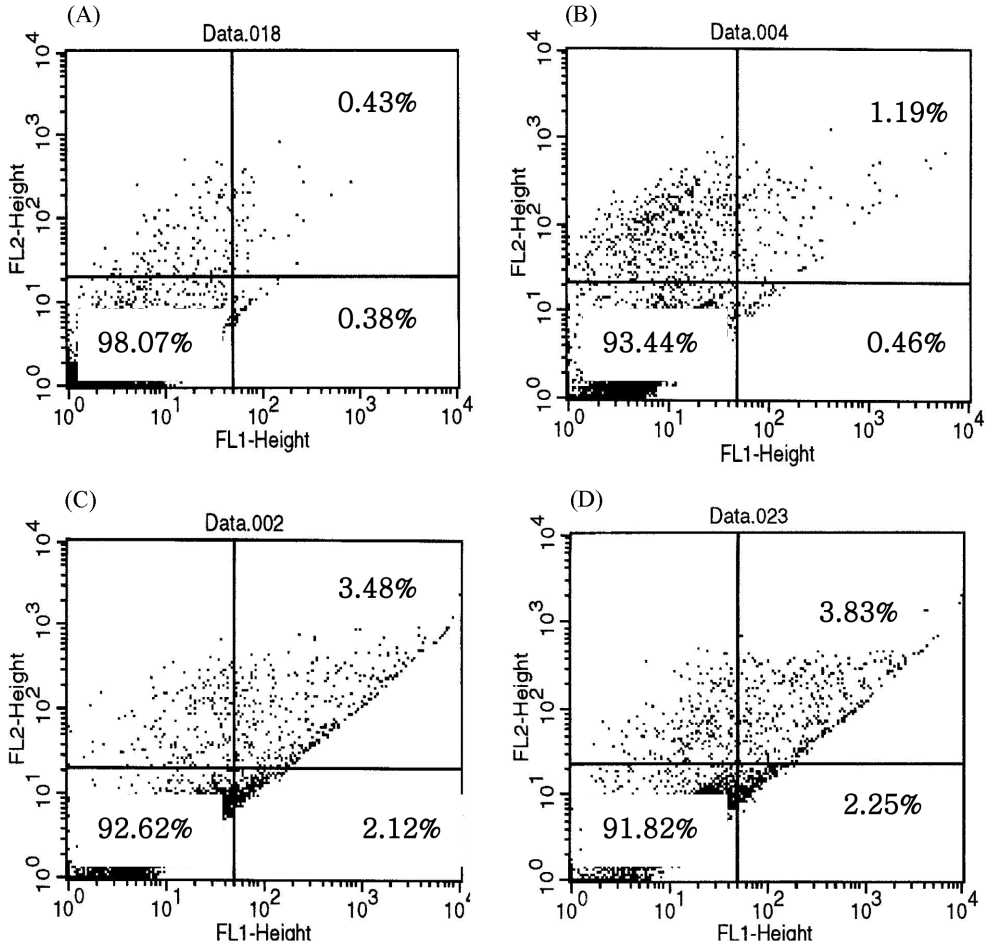

Fig. 10. Representative figures of flow cytometric detection of apoptosis in time-dependent manner. Lower right quadrants of the box (Annexin V positive and PI negative) represent percentages of apoptotic cells with preserved plasma membrane integrity, and upper right quadrants (Annexin V positive and PI positive) refer to necrotic or lately apoptotic cells with loss of plasma membrane integrity. Untreated cells were unstained with Annexin V and PI, suggesting that most of them were live cells. (X axis: Annexin V, Y axis: PI), (A) Control, (B) Rapamycin, (C) NNK 200 μM, 4 hrs, (D) Rapamycin + NNK 200 μM, 4 hrs.

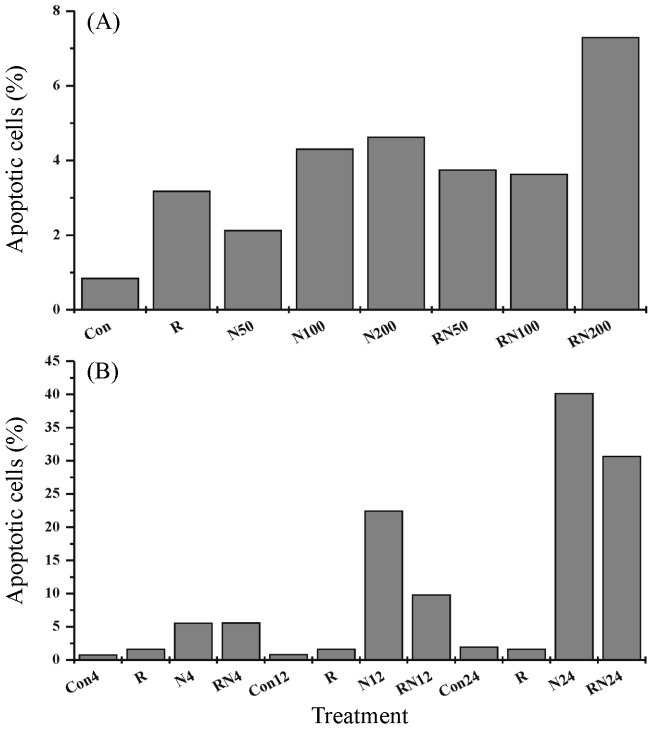

Fig. 11. Representative quantification of concentration- and time-dependent cell alterations detected by flow cytometric analysis. Apoptotic cells increased in concentration- and time-dependent manners. (A) Percentage of apoptotic cells in concentration-dependent study, (B) Percentages of apoptotic cells in time-dependent study.

with Annexin V and propidium iodide (PI), which is an important marker for distinguishing early apoptosis and necrosis. Untreated cells were not stained with Annexin V and PI, suggesting that most of them were intact live cells. NNK induced significant apoptosis in concentration- and time-dependent manners. In concentration-response study, percentage of apoptosis in NNK with rapamycin was higher than that of NNK alone, also with concentration-dependent increase pattern. In time-course study, the percentage of apoptosis in NNK alone as well as NNK+rapamycin was increased time-dependently (Representative Fig. 10 and Fig. 11 A and B).

Transient transfection assay

Changes in luciferase activity in transient transfection with bicistronic constructs

To determine the status of cap-dependent and -independent protein translation in NNK-induced apoptosis in human bronchial epithelial cells, we performed transient transfection using a bicistronic construct. Luciferase activity increased in 50 and 100 μM NNK for 2 hrs, whereas decreased in 200 μM NNK for 2 hrs. Similar pattern was detected in NNK with rapamycin. Activities were lower in NNK with rapamycin than with NNK alone (Fig. 12A), and

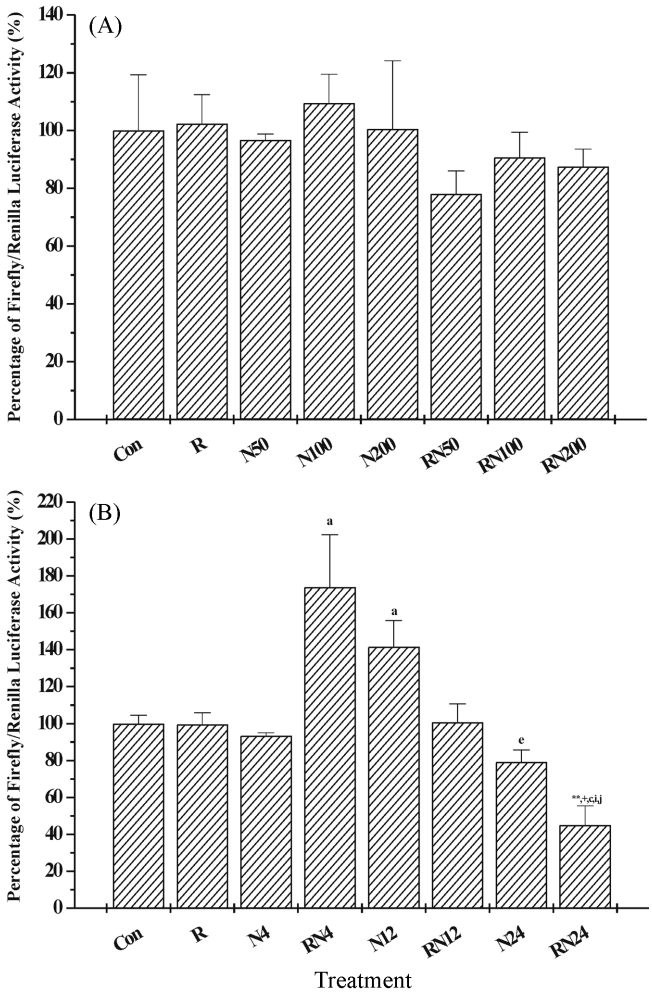

Fig. 12. Expression of luciferase from bicistronic construct (pcDNA-fLuc-polIRES-rLuc) in human bronchial epithelial cells. Luciferase activity was expressed as a percentage of fLuc/rLuc. Experiments were repeated three times, and the results represent means ± SE (n = 3), Dunnetts test for multiple comparisons and Students *t*-test. (A) Ratio of fLuc/rLuc activity in concentration-dependent manner after 2 hrs following NNK treatment. C: Control; R: Rapamycin; N50: NNK 50 μM; N100: NNK 100 μM; N200: NNK 200 μM; RN50: Rapamycin + NNK 50 μM; RN100: Rapamycin + NNK 100 μM; RN200: Rapamycin + NNK 200 μM (B) Ratio of fLuc/rLuc activity in time-dependent manner after 200 μM of NNK treatment. Statistical significance of difference from control (**$p < 0.01$), rapamycin ($^{+}p < 0.05$), NNK 4 hrs ($^{a}p < 0.05$), NNK 12 hrs ($^{e}p < 0.05$), NNK 24 hrs ($^{c}p < 0.05$), rapamycin + NNK 4 hrs ($^{i}p < 0.05$), rapamycin + NNK 12 hrs ($^{j}p < 0.05$). C: Control; R: Rapamycin; N4: NNK, 4 hrs; RN4: Rapamycin + NNK, 4 hrs; N12: NNK, 12 hrs; RN12: Rapamycin + NNK, 12 hrs; N24: NNK, 24 hrs; RN24: Rapamycin + NNK, 24 hrs.

the relative luciferase percentage (fLuc/rLuc) decreased in a time-dependent manner in NNK with rapamycin (Fig. 12B).

Luciferase activity in pGL3 Apaf-1 promoter constructs transfected cells

To understand the role of Apaf-1 in human bronchial

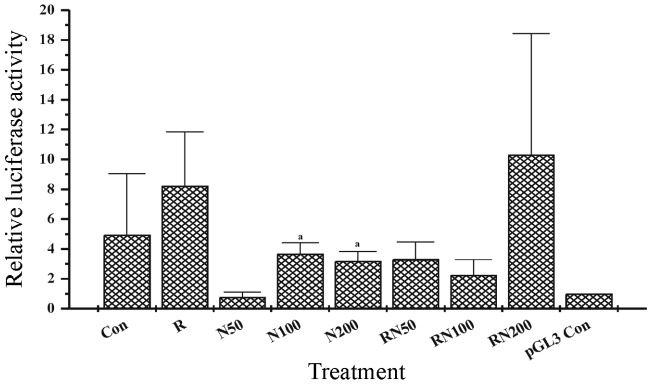

Fig. 13. Expression of pGL3 Apaf-1 promoter construct in human bronchial epithelial cells. Cells were transiently transfected and harvested. Luciferase activity was expressed as a ratio of positive control pGL3 control. Values represent as means ± SE (n = 3). Statistical significance of difference from NNK 50 μM ($^{a}p < 0.05$). Dunnetts test for multiple comparisons and Students *t*-test.

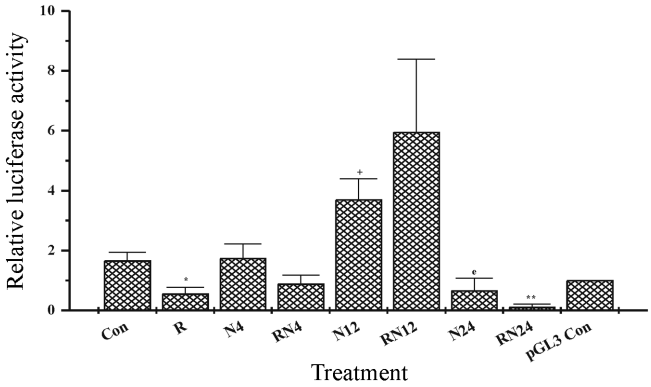

Fig. 14. Expression of pGL3 Apaf-1 promoter construct in human bronchial epithelial cells. Luciferase activity was expressed as a ratio of positive control pGL3 control. Values represent as means ± SE (n = 3). Statistical significance of difference from control ($^{*}p < 0.05$, $^{**}p < 0.01$), rapamycin ($^{+}p < 0.05$), NNK 12 hrs ($^{e}p < 0.05$). Dunnetts test for multiple comparisons and Students *t*-test.

epithelial cells, we performed transient transfection using Apaf-1 promoter construct**.** In concentration-dependent treatment, luciferase activity increased as a function of NNK concentration. Similar concentration-dependent increase pattern was observed in NNK with rapamycin (Fig. 13). In time-course treatment, the luciferase activity increased significantly in both of NNK, and NNK with rapamycin until 12 hrs, then decreased abruptly at 24 hrs (Fig. 14).

Discussion

Reduction of cap-dependent protein translation can be induced under various cellular conditions. Several apoptosis-related genes including XIAP [16], DAP5 [14], c-*myc* [31] and Apaf-1, contain IRES [12], thus, whose protein products can be translated under conditions where cap-dependent translation is inhibited. The function of FRAP in cells is potently inhibited by rapamycin [11]. Rapamycin inhibits cap-dependent, but not cap-independent translation [2,17].

In this study, we hypothesized that state of protein translation could play an important role in NNK-induced apoptosis. Because Apaf-1 has IRES, we investigated whether Apaf-1 might be associated with NNK-induced apoptosis through either cap-dependent or -independent protein translation. Our study indicated that IRES-dependent translation was critical to the initial stage of NNK-induced apoptosis. Caspase 3 and 9 have been shown to be a key component of the apoptotic machinery [8]. Caspase 3 and 9 activities showed concentration-dependent increases with NNK 2 hrs (Fig. 3), demonstrating that apoptosis induced by NNK in human bronchial epithelial cells is associated with activations of caspases 3 and 9. Our results are confirmed by Kaliberov *et al*'s study [18] that H1466 lung cancer cell study with AdVEGFBAX showed time-dependent increases of caspase 3, 8, and 9 activities at 3, 6, 9, 12, and 18 hrs. Western blotting analysis revealed similar concentration-dependent increase in the expression of caspase 3, and 9 (Fig. 2). Interestingly, rapamycin induced higher expression of caspase 3, and 9 indicating there might be different pathways for the activations of caspase 3, and 9. However, such pattern of rapamycin-dependent expression of caspase 3 and p was not obvious at later time-point. As time passed, NNK alone induced more protein expression of caspase 3 and 9, suggesting that IRES-dependent caspase 3 and 9 expression might be more responsible for NNK-induced apoptosis in this study. Recent data showed that miotochondrially-localized active caspase 3 and 9 result mostly from translocation from cytosol into the intermembrane space and partly from caspase-mediated activation in the organelle rather than Apaf-1-mediated activation [6]. Our data showed Apaf-1 protein expression level was increased by rapamycin pretretment. There were also concentration-dependent increase of Apaf-1 protein expression at early time point. However, such pattern was not found at later time-point, indicating that initial apoptosis is associated with cap-independent activation of Apaf-1. Apaf-1 localizes exclusively in the cytosol and, upon apoptotic stimulation, translocation to perinuclear area but not to the mitochondria. Several other studies also showed that during stress signaling caspase 2 activation occurred upstream of mitochondrial damage and the release of cytochrome c, suggesting that caspase 9 activation by Apaf-1 is not an initiator of the caspase cascade [1]. Fluorometric analysis of caspase 3 showed clear time-dependent increase, however, the general level of expression was much lower in rapamycin pretreatment group than those of NNK alone. Whereas such difference between caspase 3 and 9 was not

observed as found in Western blot analysis (Fig. 2 and 3). Interestingly, rapamycin also induced high expression of caspase 3, but not of caspase 9 unlike Western blot analysis strongly suggesting that increased amount of caspase 3 and 9 protein levels might not be related the practical activity.

Regradless of rapamycin pretreatment, caspase 3 activity increased initially and decreased, then increased again. However, caspase 9 activity showed somewhat different patterns. NNK alone did not induced any changes while rapamycin pretreatment caused clear concentration-dependent increase (Fig. 4). Our findings of rapamycin-induced caspase 3 activation is coincident with Nottingham *et al*'s [28] result that rapamycin expressed activated caspase 3 in spinal cord of rats. Rapamycin-dependent increase pattern of caspase 9 activities strongly suggest that caspase 9 may have IRES as Apaf-1 does. Proapoptotic Bax and Bid protein expression pattern reconfirm our interpretation. Generally, rapamycin pretreatment increased the amount of protein expression as shown in Figs. 5 and 7. Such rapamycin-dependent cytochrome c release suggest that caspase 9 activation may occur through cap-independent pathway. In contrast, anti-apoptotic Bcl2 expression was not affected by rapamycin pretretment, thus, suggesting that Bcl2 was not associated with IRES protein translation. Our results demonstrated that NNK might activate caspase-dependent apoptosis through alternative pathways during caspase-dependent apoptosis. Moreover, cytochrome c release was more prominent in NNK with rapamycin than NNK alone. Our finding is consistant with other groups result that gamma-tocopherol quinone induced apoptosis in cancer cells through caspase 9 activation and cytochrome c release [5].

In FACS analysis, NNK induced significant apoptosis while live cells decreased in concentration-, time-dependent manner (Fig. 10 and 11). These data indicated that fluorocytometric apoptosis patterns showed similar to those of caspase assay and Western blotting. Similar results were also obtained with NNK-induced apoptosis on endothelial cells stained with terminal deoxyribonucleotide transferase-mediated dUTP nick-end labelling and annexin V [29]. These data support our results that NNK caused apoptosis in concentration-, time-dependent manners and cap-independent protein translation was responsible for early apoptosis. To understand the relative roles of cap-dependent and -independent protein translations in NNK-induced apoptosis in human bronchial epithelial cells, we performed transient transfection using a bicistronic construct. In concentration-dependent treatment, the relative luciferase ratio (*f*Luc/*r*Luc) was low in NNK with rapamycin, and decreased in time-dependent manner (Fig. 12). These results showed that cap-independent translation was evident at initial stage, however, during the later stage of apoptosis, cap-dependent translation became prominent. In fact, DAP5s 5' UTR could drive cap-independent translation in reporter studies using bicisonic vectors [14]. These results support our data that NNK induced apoptosis through selective control of cap-dependent and/or -independent protein translation as a function of time. To determine the precise role of Apaf-1 in NNK-induced apoptosis in human bronchial epithelial cells, we performed transient transfection assay with pGL3 Apaf-1 promoter construct and Western blotting. As mentioned earlier, the luciferase activity was higher in NNK with rapamycin than that of NNK alone, especially at 200 mM NNK (Fig. 13) and increased significantly by 12 hrs treatment, then decreased abruptly (Fig. 14). Other study showed that the initiation of protein translation through the Apaf-1 IRES was not increased during later stages of apoptosis, probably reflecting that Apaf-1 is required for initial steps of apoptosis [25]. Taken together with above results, our data strongly suggest that IRES-dependent protein translation is responsible for early stage of NNK-induced apoptosis. Our results may be applicable as the mechanical basis of lung cancer treatment.

Acknowledgments

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