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Involvement of AMP-Activated Protein Kinase and p38 Mitogen-Activated Protein Kinase in 8-Cl-cAMP-Induced Growth Inhibition

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8-Cl-cAMP (8-chloro-cyclic AMP), which induces differentiation, growth inhibition and apoptosis in various cancer cells, has been investigated as a putative anti-cancer drug. Although we reported that 8-Cl-cAMP induces growth inhibition via p38 mitogen-activated protein kinase (MAPK) and a metabolite of 8-Cl-cAMP, 8-Cl-adenosine mediates this process, the action mechanism of 8-Cl-cAMP is still uncertain. In this study, it was found that 8-Cl-cAMP-induced growth inhibition is mediated by AMP-activated protein kinase (AMPK). 8-Cl-cAMP was shown to activate AMPK, which was also dependent on the metabolic degradation of 8-Cl-cAMP. A potent agonist of AMPK, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) could also induce growth inhibition and apoptosis. To further delineate the role of AMPK in 8-Cl-cAMP-induced growth inhibition and apoptosis, we used two approaches: pharmacological inhibition of the enzyme with compound C and expression of a dominant negative mutant (a kinase-dead form of AMPKα2, KD-AMPK). AICAR was able to activate p38 MAPK and pre-treatment with AMPK inhibitor or expression of KD-AMPK blocked this p38 MAPK activation. Cell growth inhibition was also attenuated. Furthermore, p38 MAPK inhibitor attenuated 8-Cl-cAMP- or AICAR-induced growth inhibition but had no effect on AMPK activation. These results demonstrate that 8-Cl-cAMP induced growth inhibition through AMPK activation and p38 MAPK acts downstream of AMPK in this signaling pathway.

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8-Chloro-cyclic AMP (8-Cl-cAMP)[1] is known to show potent growth inhibitory effect as well as reverse-transforming activity in cancer cells, and accordingly, it is under investigation for the anti-cancer therapeutic potential (Tortora et al., 1995; McDaid and Johnston, 1999; Propper et al., 1999). Previously, it was found that 8-Cl-cAMP could modulate the ratio of regulatory (R) subunits (type I or II) of cAMP-dependent protein kinase (PKA) (Cho-Chung et al., 1989). Type I-R subunit that has a stimulatory effect on the cellular proliferation was observed to decrease after the incubation with 8-Cl-cAMP, which might result in the growth inhibition (Beebe et al., 1989; Cho-Chung et al., 1989). However, another explanation for 8-Cl-cAMP-induced growth inhibition has been presented by several research groups (Langeveld et al., 1997; Halgren et al., 1998; Gandhi et al., 2001; Lamb and Steinberg, 2002). They suggested that it is not 8-Cl-cAMP itself but 8-Cl-adenosine, one of the metabolites of 8-Cl-cAMP, which actually exerts growth inhibitory effect (Langeveld et al., 1997; Gandhi et al., 2001). Chemical inhibitors of adenosine kinase and adenosine transporter attenuated the 8-Cl-cAMP-induced growth inhibition, which means that metabolic conversion of 8-Cl-cAMP into 8-Cl-adenosine or further metabolites is indispensable for the cytotoxic activity of 8-Cl-cAMP (Halgren et al., 1998; Lamb and Steinberg, 2002). We also reported that 8-Cl-cAMP should be converted to further metabolites for its growth inhibitory action, and 8-Cl-cAMP and 8-Cl-adenosine induce growth inhibition through the same mechanisms, that is, protein kinase C and p38 mitogen-activated protein kinase (MAPK) activation (Ahn et al., 2004, 2005).

Metabolic conversion of 8-Cl-cAMP might result in the accumulation of the metabolites such as 8-Cl-AMP and 8-Cl-ATP, which could disturb the intracellular AMP or ATP pool. In fact, it was shown that the cellular concentration of ATP decreased and that of 8-Cl-ATP increased following the treatment with 8-Cl-cAMP or 8-Cl-adenosine (Gandhi et al., 2001). ATP depletion means the increase of AMP/ATP ratio that is the stimulatory signal for AMP-activated protein kinase (AMPK) (Hardie, 2003; Carling, 2004). In the present study, we tested if AMPK activation could take part in the signaling mechanisms of 8-Cl-cAMP-induced growth inhibition.

AMPK is an intracellular sensor of ATP level, more precisely AMP/ATP ratio, and has a crucial role in maintaining the energy balance, which is very important for the cell survival (Carling, 2004). It is activated when cellular ATP is depleted, and is known to turn off the ATP consuming pathways such as fatty

Abbreviations: 8-Cl-cAMP, 8-chloroadenosine-3′,5′-cyclic monophosphate; MAPK, mitogen-activated protein kinase; AMPK, AMP-activated protein kinase; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; KD-AMPK, kinase-dead form of AMPKα2; PKA, cAMP-dependent protein kinase; AMPKKs, AMPK kinases; CaMKK, Ca^{2+}/calmodulin-dependent protein kinase kinase; PI, propidium iodide; NBTI, nitrobenzyl thioinosine.

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acid synthesis, lipolysis, and protein synthesis, and turn on the ATP generating pathways such as glycolysis, fatty acid oxidation, and glucose uptake (Hardie, 2003; Carling, 2004). AMPK is a heterotrimeric complex composed of a catalytic (α) and two R (β and γ) subunits. The α subunit possesses Ser/Thr protein kinase activity that is stimulated after the phosphorylation by upstream kinases, AMPK kinases (AMPKKs). The β subunit is known to be a scaffold for the trimeric complex, and the γ subunit is regarded to contain the AMP-binding domains. AMP-binding to the γ subunit induces the enzymatic activity of the α subunit by means of making it more susceptible to AMPKK. LKB1 (Woods et al., 2003; Shaw et al., 2004) and Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) (Hurley et al., 2005) have been suggested as putative AMPKKs. Besides the AMP/ATP ratio, AMPK can also be activated by osmotic stress (Patel et al., 2001), anti-diabetic drugs (Fryer et al., 2002), and 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) (Corton et al., 1995).

AICAR has been widely used as a potent AMPK activator. It is converted to ZMP, an analog of AMP, by the action of adenosine kinase after entering the cells (Corton et al., 1995; Hardie, 2003). When treated to various cells including vascular smooth muscle cells (Nagata et al., 2004; Igata et al., 2005), adipocytes (Dagon et al., 2006), pancreatic beta cells (Kefas et al., 2003), and other cancer cells (Imamura et al., 2001; Xiang et al., 2004; Rattan et al., 2005), AICAR induced growth inhibition and apoptosis, which means that AMPK can be the key regulator of cellular growth. Therefore, we decided to look at the role(s) of AMPK activation in the signaling mechanisms of 8-Cl-cAMP-induced growth inhibition.

Materials and Methods

Reagents

8-Cl-cAMP, 8-Cl-adenosine, 8-Cl-AMP, 8-Cl-ATP, and Sp-8-Cl-cAMPS were purchased from Biolog (Bremen, Germany). AICAR was from Toronto Research Chemicals (Ontario, Canada). SB203580 was obtained from A.G. Scientific (San Diego, CA). Compound C was from Calbiochem (San Diego, CA). A134974, ABT-702, and NBTI were purchased from Sigma–Aldrich (St. Louis, MO). Propidium iodide (PI) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were also purchased from Sigma–Aldrich. [γ-^{32}P]-ATP (6,000 Ci/mmol) was purchased from Dupont NEN (Boston, MA). Transfection reagent, Lipofectamine™ 2000 was from Invitrogen (Carlsbad, CA). Dominant negative expression vector with kinase-dead (KD) form of AMPKα2 was a kind gift from Dr. Morris J. Birnbaum (Howard Hughes Medical Institute, University of Pennsylvania Medical School, Philadelphia, PA) (Mu et al., 2001).

Cell culture

HeLa (cervical carcinoma), MCF7 (breast carcinoma), and MDA-MB-231 (breast carcinoma) cells were maintained in Eagle's minimal essential medium (HyClone, Logan, UT), and K562 (leukemia) and HL60 (acute promyelocytic leukemia) cells were cultured in RPMI1640 (HyClone). DT (K-*ras*-transformed NIH3T3) cells were grown in Dulbecco's modified Eagle medium (HyClone). Each culture medium was supplemented with 10% fetal bovine serum (HyClone), 100 units/ml penicillin G, and 100 μg/ml streptomycin. Cell number or viability was determined with Coulter™ counter (Beckman Coulter, Fullerton, CA) or MTT assay as described before (Ahn et al., 2004). To observe dead cells, cells were stained with PI (50 μg/ml), and then observed under Optiphot-2 fluorescence microscope (Nikon, Tokyo, Japan). To investigate cell morphology, cells were stained with Acridine orange (10 μg/ml), and the cells were observed as above.

Construction of stable cell line

1.5×10^5 HeLa cells were seeded into 60mm dish in Eagle's minimal essential medium without serum and KD-AMPK expression vector and pcDNA3.0 vector (mock) were transfected into cells after 24 h using Lipofectamine™ 2000 according to the manufacturer's protocol. It was then selected in the presence of G418 antibiotic (1 mg/ml) for 3 weeks.

Western blotting

Cells were harvested by centrifugation and washed with PBS. The cell pellet was suspended in an extraction buffer (25 mM Tris–Cl, pH 8.0, 0.5 mM EDTA, 0.5 mM EGTA, 0.05% Triton X-100, 10 mM β-mercaptoethanol, 100 μM PMSF, 0.1mM sodium pyrophosphate, 2.5 mM NaF, 1 μg/ml each of Na_3VO_4, benzoamidin, aprotinin, antipain, and leupeptin) and incubated on ice for 15 min. After centrifugation at 14,000*g* for 15 min at 4°C, the supernatant was taken as cell extract. The extracts were separated on 10% SDS–PAGE and transferred onto a PVDF membrane. The protein-bound membrane was incubated with the appropriate antibodies followed by horse-radish peroxidase-conjugated anti-mouse or rabbit IgG antibody (Bio-Rad, Hercules, CA). The relevant protein bands were then visualized using the ECL™ detection kit (Amersham, Piscataway, NJ). Phospho-AMPKα (Thr172) and total AMPKα antibodies were from Cell Signaling Technology (Danvers, MA). Antibody for phospho-p38 MAPK (Thr180/Tyr182) was obtained from BD Transduction Laboratories (San Diego, CA), and total p38 MAPK antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Also, antibodies for c-myc and actin were purchased from Santa Cruz Biotechnology.

AMPK activity assay

HeLa cell lysate was precipitated with saturated ammonium sulfate solution. Protein (2 μg) was incubated in a reaction buffer (40 mmol/L HEPES, pH 7.0; 80 mmol/L NaCl; 5 mmol/L magnesium acetate; 1 mmol/L DTT; 200 μmol/L AMP and ATP; 2 μCi [γ-^{32}P]-ATP) with 200 μmol/L SAMS peptide (synthesized by Peptron, Daejeon, Korea) for 10 min at 30°C. The reaction mixtures were then spotted onto P81 phosphocellulose disks (Whatman, Maidstone, UK), and washed three times with 1% phosphoric acid. Disks were air-dried, and radioactivity was measured with liquid scintillation counter (Wallac, Turku, Finland).

DNA fragmentation assay

Harvested cells were suspended in lysis buffer (5 mmol/L Tris–Cl, pH 7.4; 20 mmol/L EDTA; 0.5% Triton X-100), and incubated on ice for 30 min. After centrifugation at 14,000*g* for 30 min at 4°C, fragmented DNA in the supernatant was purified by phenol/chloroform extraction and precipitated with ethanol. DNA was then electrophoresed on 2% agarose gel and visualized by staining with Ethidium bromide.

Flow cytometry

Cells were fixed with ice-cold 70% ethanol for at least 3 h, and then stained with PI (50 μg/ml) containing RNase A (50 μg/ml) at 37°C for 30 min. DNA content was analyzed by FACS-Calibur™ flow cytometer (BD Bioscience, San Jose, CA) using CellQuest™ programs (BD Bioscience).

Results

AMPK activity increased after 8-Cl-cAMP treatment

It was reported that cellular ATP was depleted when 8-Cl-cAMP or 8-Cl-adenosine was treated to multiple myeloma cells (Gandhi et al., 2001). Decrease of intracellular ATP concentration can result in the activation of AMPK. To verify whether 8-Cl-cAMP is able to induce the AMPK activation, we tried to measure cellular AMPK activity after treating the cells

with 8-Cl-cAMP. AMPK activation can be assessed by Western blotting with the antibody against phosphorylated AMPK α subunit (Thr-172) or by kinase assay using a specific substrate, SAMS peptide (Witters and Kemp, 1992).

HeLa cells were incubated with 8-Cl-cAMP for 3 days, and Western blotting was performed to measure the phosphorylation of AMPK (Fig. 1A). AMPK phosphorylation started to increase 36–48 h after 8-Cl-cAMP treatment, in a time-dependent manner. Also, in the kinase assay, increase of AMPK activity was observed from 2 days after the 8-Cl-cAMP treatment (Fig. 1B). AICAR, a potent AMPK activator, was used as a positive control, and after 30 min of incubation, it could activate the AMPK.

Previously, we reported 8-Cl-cAMP-induced growth inhibition was dependent on its metabolic conversion (Ahn et al., 2004). To test if 8-Cl-cAMP-induced AMPK activation is also dependent on the metabolic degradation, metabolites of 8-Cl-cAMP such as 8-Cl-adenosine (Ado), 8-Cl-AMP (AMP), and 8-Cl-ATP (ATP) were treated to HeLa cells for 3 days, and then the level of phospho-AMPK was measured (Fig. 1C). As expected, all the metabolites could increase AMPK phosphorylation. However, an unhydrolyzable analogue of 8-Cl-cAMP, Sp-8-Cl-cAMPS (Sp-8Cl; 8-chloroadenosine-3′, 5′-cyclic monophosphorothioate, Sp-isomer), which is not influenced by the phosphodiesterase (Yokozaki et al., 1992), could not activate AMPK. When, HeLa cells were incubated with 8-Cl-adenosine for 3 days, the phosphorylation of AMPK started to increase 48 h after the treatment (Fig. 1D).

Furthermore, an adenosine kinase inhibitor (A134974) and an adenosine uptake inhibitor (NBTI; S-(4-nitrobenzyl)-6-thioinosine) effectively attenuated the AMPK phosphorylation induced by 8-Cl-cAMP (Fig. 1E). These data clearly demonstrate that 8-Cl-cAMP must be converted to its metabolites in order to induce the activation of AMPK.

AICAR induced growth inhibition and apoptosis

Next, we wanted to check whether AMPK activation by 8-Cl-cAMP is related with growth inhibition. We examined the effect of AICAR, an AMPK activator, on cellular growth. As reported previously (Imamura et al., 2001; Xiang et al., 2004; Rattan et al., 2005), AICAR showed growth inhibitory effect on various cancer cells such as HeLa cells, MCF7 cells, MDA-MB-231 cells, and K562 cells in a dose-dependent manner (Fig. 2A). In HeLa cells, cell number decreased below 40% of control following incubation with 200 μmol/L AICAR for 5 days (Fig. 2B). And this decrease of cell number was mainly caused by apoptotic cell death, which was assessed by PI staining for dead cells (Fig. 2C), chromosomal DNA fragmentation assay (Fig. 2D), and flow Cytometric analysis of DNA content (Fig. 2E). AICAR must be phosphorylated to ZMP by adenosine kinases in order to activate AMPK (Hardie, 2003). Accordingly, when HeLa cells were pre-incubated with an adenosine kinase inhibitor (A134974), AICAR could not induce apoptosis (Fig. 2E). Also, when HL60 cells were co-treated with AICAR and ABT-702, another

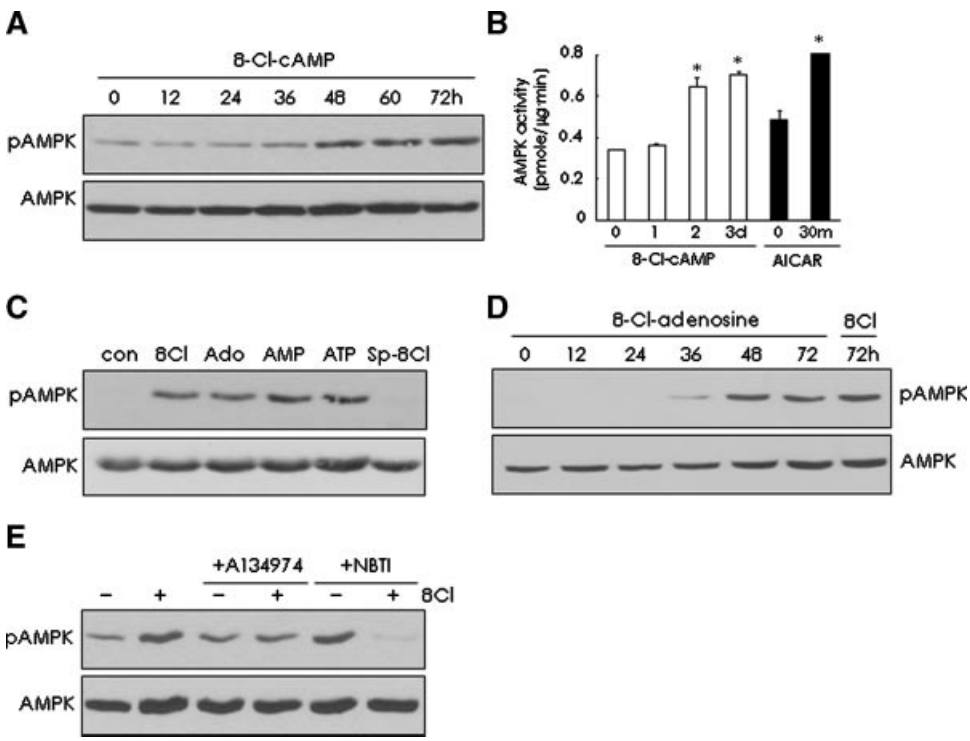

Fig. 1. AMPK activity increased after 8-Cl-cAMP treatment. A: HeLa cells were treated with 8-Cl-cAMP (10 μmol/L) for the indicated times, and phospho- or total AMPK Western blotting was carried out. B: After 8-Cl-cAMP (10 μmol/L) or AICAR (2 mmol/L) treatment, AMPK activity assay was performed using SAMS peptide as substrate. Bars denote AMPK enzymatic activity (mean + SD, n = 3). Asterisks mean difference from control group ($P < 0.01$, two-tailed t-test). C: HeLa cells were treated with 8-Cl-cAMP (8Cl, 10 μmol/L), 8-Cl-adenosine (Ado, 10 μmol/L), 8-Cl-AMP (AMP, 10 μmol/L), 8-Cl-ATP (ATP, 10 μmol/L), and Sp-8-Cl-cAMPS (Sp-8Cl, 10 μmol/L) for 3 days, and then phospho- or total AMPK Western blotting was carried out. D: 8-Cl-adenosine (10 μmol/L) and 8-Cl-cAMP (8Cl, 10 μmol/L) were treated to HeLa cells for the indicated times, and phospho- or total AMPK Western blotting was carried out as in C. E: 8-Cl-cAMP was treated to HeLa cells for 3 days in the presence or absence of A134974 (an adenosine kinase inhibitor, 10 μmol/L) and NBTI (an adenosine transporter inhibitor, 100 μmol/L). Cells were lysed and Western blotting was performed as in C.

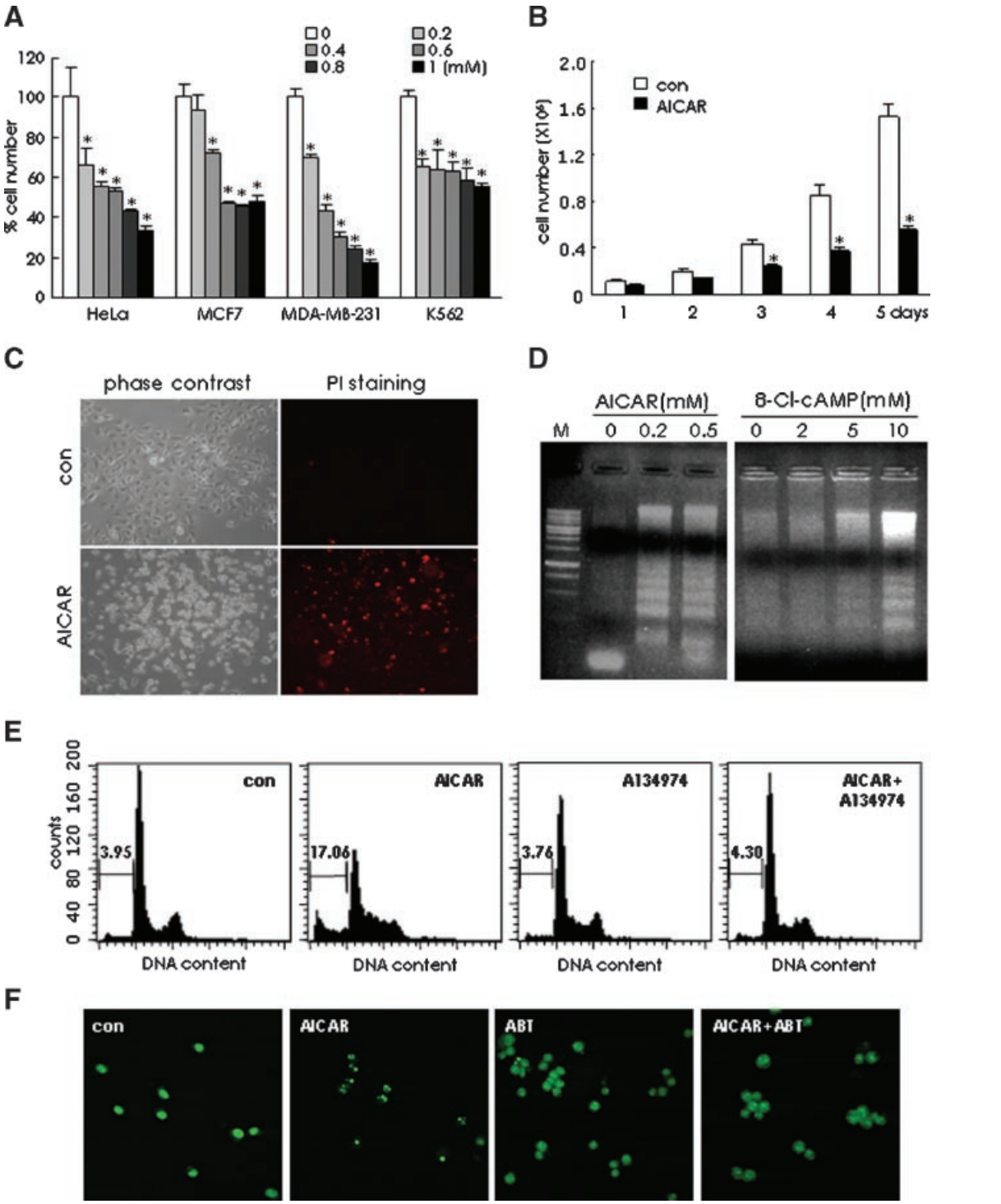

Fig. 2. AICAR induced growth inhibition and apoptosis. A: HeLa (cervical carcinoma), MCF7 (breast carcinoma), MDA-MB-231 (breast carcinoma), and K562 (leukemia) cells were incubated with AICAR (0, 0.2, 0.4, 0.6, 0.8, and 1 mmol/L) for 3 days, and then MTT assay (for HeLa, MCF7, and MDA-MB-231) or cell counting (for K562) was carried out. Bars denote cell number normalized to control group (mean + SD, n = 4). Asterisks mean difference from control ($P < 0.01$, two-tailed *t*-test). B: HeLa cells were treated with AICAR (200 μmol/L), and then cell number (mean + SD, n = 3) was counted everyday until Day 5. Asterisks mean difference from control ($P < 0.01$, two-tailed *t*-test). C: HeLa cells were incubated with AICAR (2 mmol/L) for 3 days, and PI staining was performed to visualize the dead cells. Pictures were taken under 400× magnification. D: After incubation with AICAR or 8-Cl-cAMP for 3 days, chromosomal DNA was extracted to check the apoptotic DNA fragmentation. E: HeLa cells were treated with AICAR (200 μmol/L) in the presence or absence of A134974 (10 μmol/L) for 3 days. After PI staining, flow cytometry was carried out to analyze DNA content. Inserted numbers represent the percentage of apoptotic cell population (less than 2N DNA content). F: HL60 cells were treated with AICAR (200 μmol/L) in the presence or absence of ABT-702 (ABT, 10 μmol/L) for 3 days and stained with acridine orange. Pictures were taken under 400× magnification.

adenosine kinase inhibitor, apoptosis was attenuated as judged by acridine orange staining (Fig. 2F).

AMPK could activate p38 MAPK during 8-Cl-cAMP or AICAR-induced growth inhibition

We previously showed that p38 MAPK was activated by 8-Cl-cAMP and it could play a critical role during 8-Cl-cAMP-induced growth inhibition (Ahn et al., 2005). If AMPK activation is also important for the action of 8-Cl-cAMP, there should be significant relationship between AMPK activation and p38 MAPK activation. In addition to the activation of AMPK, AICAR could also activate p38 MAPK in a time-dependent manner in HeLa cells (Fig. 3A). This activation of p38 MAPK was diminished by the treatment with A134974 and NBTI (Fig. 3B). 8-Cl-cAMP-induced p38 MAPK phosphorylation was also downregulated by A134974 and NBTI (Fig. 3C). Besides, a selective AMPK inhibitor, compound C (McCullough et al., 2005) completely abolished not only the activation of AMPK but also the p38 MAPK phosphorylation (Fig. 3D). 8-Cl-cAMP-induced AMPK and p38 MAPK activation was also blocked by the pre-treatment with compound C (Fig. 3E).

However, even in the presence of a p38 MAPK inhibitor, SB203580, AICAR could still activate AMPK while it clearly blocked the p38 MAPK activation (Fig. 3F), suggesting that AMPK activation occurs upstream of p38 MAPK activation.

p38 MAPK inhibitor, SB203580, blocked AICAR-induced growth inhibition and apoptosis

Previously, we showed that SB203580 blocked the 8-Cl-cAMP-induced growth inhibition and apoptosis (Ahn et al., 2005). Accordingly, if p38 MAPK mediates AICAR-induced growth inhibition and apoptosis, then the inhibitor should

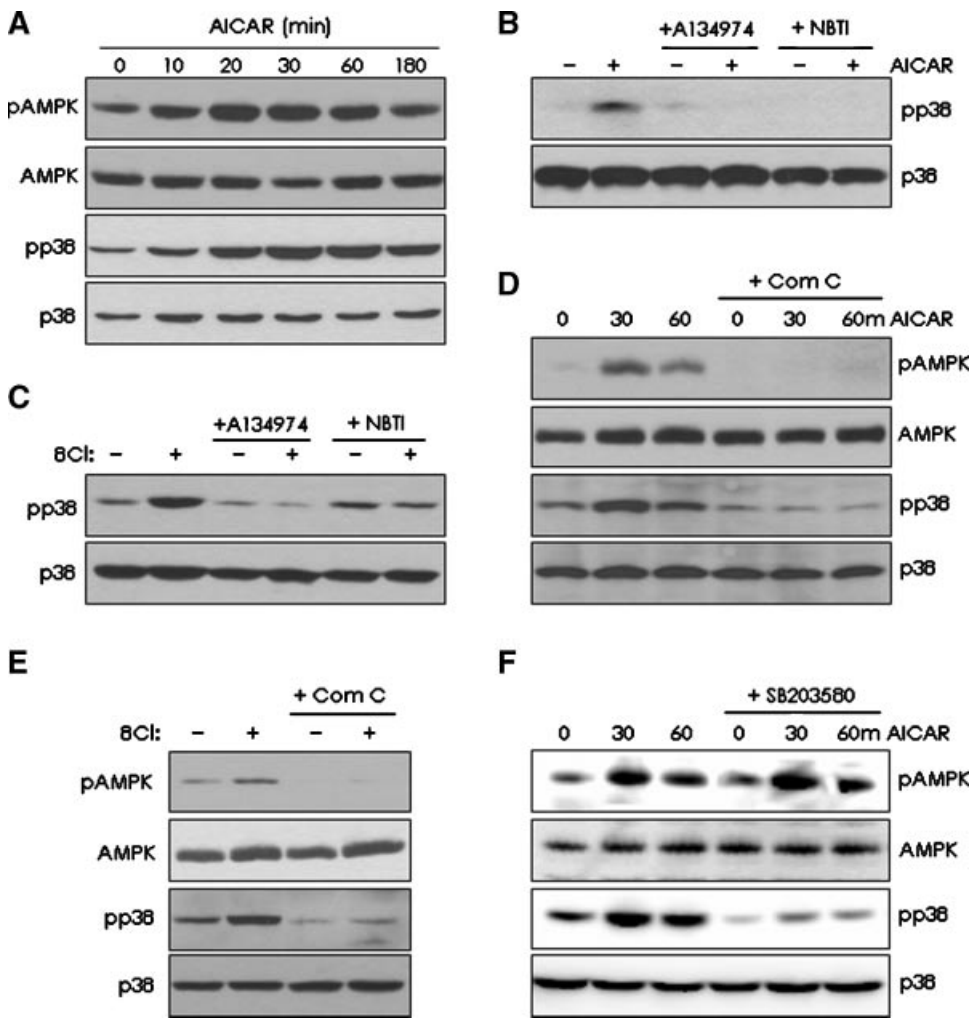

Fig. 3. AMPK could activate p38 MAPK. A: After HeLa cells were treated with AICAR (2 mmol/L) for 3 h, AMPK and p38 MAPK activation was measured by Western blotting with phospho-specific antibodies. B: HeLa cells were pretreated with A134974 (10 μmol/L) or NBTI (100 μmol/L) for 1 h prior to AICAR (30 min, 2 mmol/L) treatment, and then Western blotting was performed with phospho- and total p38 MAPK antibodies. C: HeLa cells were incubated with 8-Cl-cAMP (8Cl, 10 μmol/L) for 3 days with or without A134974 (10 μmol/L) or NBTI (100 μmol/L), and then Western blotting was performed with phospho- and total p38 MAPK antibodies as in B. D: HeLa cells were pretreated with compound C (AMPK inhibitor; 20 μmol/L) for 1 h prior to AICAR treatment. Cells were lysed and Western blotting was performed as in A. E: HeLa cells were incubated with 8-Cl-cAMP (8Cl, 10 μmol/L) for 3 days with or without compound C (2 μmol/L). Cells were lysed and Western blotting was performed as in A. F: HeLa cells were pretreated with SB203580 (10 μmol/L) for 1 h prior to AICAR treatment, and then cell lysates were immunoblotted with phospho-specific antibodies as in A.

also be capable of blocking the effect. As a matter of fact, AICAR-induced growth inhibition was attenuated by ABT-702, an adenosine kinase inhibitor and a p38 MAPK inhibitor, SB203580 was also able to lessen the growth inhibitory effect of AICAR (Fig. 4A). Furthermore, the inhibitory effect of SB203580 on AICAR-induced growth inhibition was apparent in the mouse fibroblast DT cells (Fig. 4B) and apoptosis in HL60 cells was also blocked by pre-treatment with SB203580 (Fig. 4C).

Kinase dead (KD)-AMPK inhibits 8-Cl-cAMP-induced growth inhibition and p38 MAPK activation

To further ascertain the role of AMPK in growth inhibition and p38 MAPK activation, we generated HeLa cell line, which overexpresses KD form of AMPKα2. A rat AMPKα2 cDNA carrying a myc epitope tag at its 5′ end was utilized where the lysine residue critical for ATP binding and hydrolysis was changed to arginine, resulting in a KD protein (Mu et al., 2001). After transfection of this KD-AMPK into HeLa cell, we established stable cell line expressing KD-AMPK. Sufficient expression of the c-myc-tagged transgene in KD cell line was confirmed by Western blotting (Fig. 5A). Next, we examined the effect of the KD-AMPK on 8-Cl-cAMP-treated cellular growth. KD-AMPK and mock expressing cell line were treated with 8-Cl-cAMP for 5 days, and were compared with normal cell growth as a control without 8-Cl-cAMP. As expected, KD-AMPK expressing cell line attenuated the effect of 8-Cl-cAMP-induced growth inhibition (Fig. 5B). Also, p38 MAPK activation by AICAR or 8-Cl-cAMP was blocked in KD-AMPK cell line (Fig. 5C,D). Based on these results, we suggest that AMPK activation occurs upstream of p38 MAPK activation in the signaling pathway of 8-Cl-cAMP- or AICAR-induced growth inhibition and apoptosis.

Discussion

8-Cl-cAMP has been studied as a prospective anti-cancer agent for many years because of its growth inhibitory effect in numerous cancer cells (Cho-Chung et al., 1989; Tortora et al., 1995; McDaid and Johnston, 1999; Propper et al., 1999). Cho-Chung et al. suggested in a series of studies that differential regulation of PKA isozymes, PKA type I and II, is the major cause of the 8-Cl-cAMP-induced anti-cancer activity (Cho-Chung et al., 1989; Tortora et al., 1990; Cho-Chung, 1992). However, Lamb and Steinberg (2002) reported that 8-Cl-cAMP's effects are unrelated to PKA-R subunit expression; instead, metabolic conversion of 8-Cl-cAMP to 8-Cl-adenosine is indispensable for its cellular toxicity. 8-Cl-cAMP can be converted to 8-Cl-adenosine by the actions of phosphodiesterases and nucleotide phosphatases in the active serum, and 8-Cl-adenosine is further metabolized into 8-Cl-AMP or 8-Cl-ATP by adenosine kinases (Gandhi et al., 2001). When

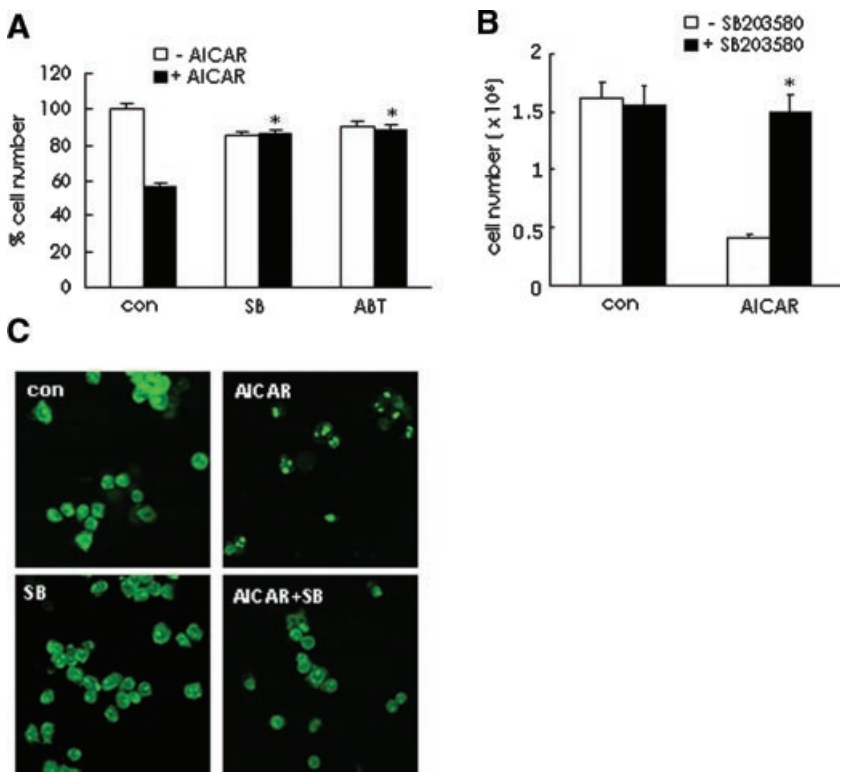

Fig. 4. p38 MAPK inhibitor blocked AICAR-induced growth inhibition and apoptosis. A: HeLa cells were treated with AICAR (200 μmol/L) for 3 days in the presence or absence of SB203580 (SB, 10 μmol/L) or ABT-702 (ABT, 10 μmol/L), and MTT assay was carried out. Bars denote cell viability normalized to control group (mean + SD, n = 4). Asterisks mean difference from AICAR-treated group ($P < 0.01$, two-tailed *t*-test). B: DT cells were treated with AICAR (200 μmol/L) for 3 days in the presence or absence of SB203580 (SB, 10 μmol/L), and MTT assay was carried out. Bars indicate mean from four independent experiments (mean + SD, n = 4). Asterisks mean difference from AICAR-treated group ($P < 0.01$, two-tailed *t*-test). C: HL60 cells were treated with AICAR (200 μmol/L) in the presence or absence of SB203580 (SB, 10 μmol/L) for 3 days and stained with acridine orange. Pictures were taken under 400× magnification.

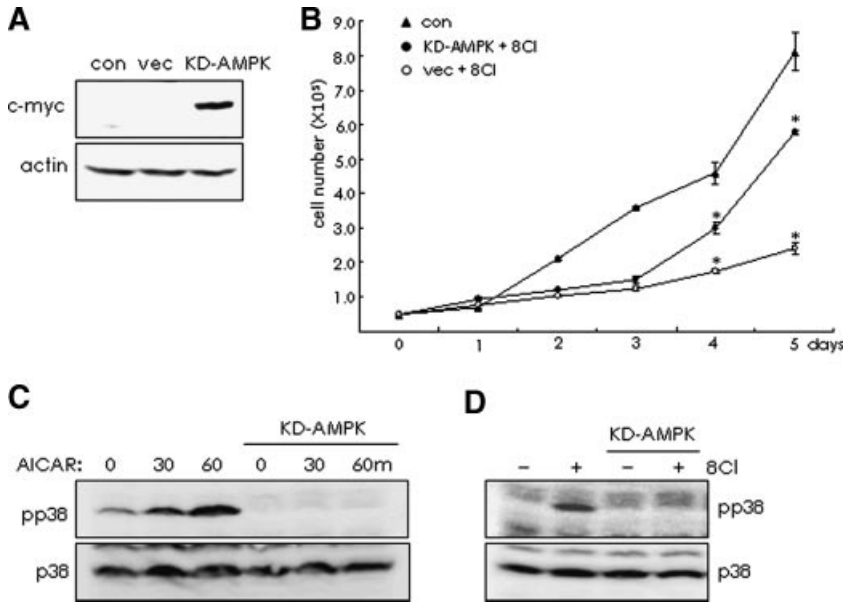

Fig. 5. Kinase-dead (KD) AMPK inhibits 8-Cl-cAMP-induced growth inhibition and p38 MAPK activation. A: HeLa cells (con), pcDNA3.0 (mock) transfected stable cells (vec), and KD-AMPK transfected stable cells (KD-AMPK) were lysed and sufficient expression of this transgene in KD cell line was confirmed by Western blotting using c-myc antibody. B: KD-AMPK and pcDNA3.0 (mock) transfected stable cells were treated with 8-Cl-cAMP (10 μmol/L), and the normal cell growth without 8-Cl-cAMP treatment was measured as a control. The cell number (mean + SD, n = 6) was counted everyday until Day 5. Asterisks mean difference from control ($P < 0.01$, two tailed *t*-test). C: HeLa cells and KD-AMPK transfected stable cells were treated with AICAR (2 mmol/L) for indicated times and p38 MAPK activation was measured by Western blotting with phospho- and total p38 MAPK antibodies. D: HeLa cells and KD-AMPK transfected stable cells were incubated with 8-Cl-cAMP (10 μmol/L) for 3 days and Western blotting was performed as in C.

serum was heat-inactivated, 8-Cl-cAMP's growth inhibitory effect was blocked in DT and HeLa cells, whereas 8-Cl-adenosine-induced growth inhibition was not affected (data not shown), implying that 8-Cl-cAMP's conversion to metabolites is indispensable in this process. Also, it was reported that 8-Cl-cAMP-induced apoptosis is mediated by p38 MAPK activity, which is also dependent on 8-Cl-cAMP's metabolic pathway (Ahn et al., 2005).

In this report, we have demonstrated that AMPK phosphorylation (Fig. 1A) and enzymatic activity (Fig. 1B) was induced by 8-Cl-cAMP, which was dependent on the metabolic conversion of 8-Cl-cAMP. Both an adenosine kinase inhibitor (A134974) and an adenosine transport inhibitor (NBTI) blocked AMPK activation (Fig. 1E), and 8-Cl-adenosine could also activate AMPK (Fig. 1D). These data are the additional evidences for the previous suggestion that the cellular functions of 8-Cl-cAMP are mediated by its metabolites (Langeveld et al., 1997; Gandhi et al., 2001; Ahn et al., 2004).

We also observed that AMPK activation could be the inhibitory signal for cellular growth. A potent AMPK activator, AICAR induced growth inhibition and apoptosis (Fig. 2), and it could activate p38 MAPK (Fig. 3A,B), implying that both 8-Cl-cAMP and AICAR share the same signaling pathway while exerting their cellular growth inhibitory effects. When co-treated with an adenosine kinase inhibitor (Figs. 2E,F, 3B, and 4A), AICAR could not induce AMPK/p38 MAPK activation and growth inhibition because AICAR should be phosphorylated to ZMP by adenosine kinases for the intracellular actions (Corton et al., 1995; Hardie, 2003).

Many investigators have demonstrated that AMPK is obviously involved in the modulation of cellular growth. It is suggested that very diverse signaling mechanisms are involved with growth inhibition and apoptosis induced by AMPK activation, for example, p53 and p21 up-regulation (Imamura et al., 2001; Xiang et al., 2004; Igata et al., 2005; Rattan et al., 2005), ERK inhibition (Nagata et al., 2004) and JNK activation (Meisse et al., 2002; Jung et al., 2004), NFκB activation (Jung et al., 2004), and Akt inhibition (Rattan et al., 2005). We and Kefas et al. (2003) showed that p38 MAPK is another signaling mediator, whereas several researchers reported that p38 MAPK had no effect on AICAR-induced growth inhibition (Meisse et al., 2002; Jung et al., 2004; Nagata et al., 2004). This discordance may be resulted from cell line specific signaling pathway of AICAR-induced growth inhibition. Nevertheless, in many cases, p38 MAPK activation was known to be associated with the cellular actions of AMPK. In ischemic heart, AMPK can activate p38 MAPK (Li et al., 2005), and AMPK stimulates VEGF expression and angiogenesis through p38 MAPK activation in skeletal muscle (Ouchi et al., 2005). p38 MAPK also participates in the stimulation of glucose uptake by AMPK (Xi et al., 2001; Pelletier et al., 2005). Similar to these observations, p38 MAPK can have a significant role during growth inhibition and apoptosis caused by AMPK activation.

Compound C, a selective inhibitor of AMPK, completely blocked AMPK/p38 MAPK activation induced by 8-Cl-cAMP or AICAR (Fig. 3D,E). But we could not investigate the effect of Compound C on 8-Cl-cAMP-induced growth inhibition because compound C itself caused severe cell death when treated for more than 2 days (data not shown). Therefore, we used dominant negative mutant with KD form of AMPK to investigate the effect on 8-Cl-cAMP-induced growth inhibition. KD-AMPK-overexpressed HeLa cell line recovered from the 8-Cl-cAMP-induced growth inhibition compared to vector only HeLa cell line (Fig. 5B). Recovery was more apparent when 8-Cl-cAMP treatment was more than 3 days. Also, KD-AMPK-overexpressed cell line blocked the p38 MAPK activation induced by 8-Cl-cAMP or AICAR (Fig. 5C,D), which is also consistent with the effect of compound C treatment.

Previously, there was a report showing by HPLC analysis that ATP concentration decreased after 8-Cl-cAMP treatment (Gandhi et al., 2001). We also have tested whether intracellular ATP was depleted after 8-Cl-cAMP treatment, which might be causative of AMPK activation (data not shown). We used an ATP assay kit based on luciferase activity that requires ATP for the optimal activation (Lu et al., 2000). When ATP level was measured using bioluminescence assay in HeLa cells, cellular ATP concentration has decreased to 70% of control level 1 day after treatment with 10 μmol/L 8-Cl-cAMP (data not shown). Cellular ATP depletion was also blocked by A134974 (data not shown), implying 8-Cl-cAMP-induced ATP depletion is dependent upon 8-Cl-cAMP's metabolic alteration.

Some researchers reported that AICAR could not activate AMPK in HeLa cells or other LKB1-deficient cells (Shaw et al., 2004; Hurley et al., 2005). But, we could observe AMPK activation by AICAR in HeLa cells as well as in MCF7 cells that are LKB1-positive (data not shown). LKB1, a Ser/Thr kinase mutated in Peutz-Jeghers syndrome patients (Hemminki et al., 1998), has been identified as the upstream kinase of AMPK (Woods et al., 2003; Shaw et al., 2004). However, it is still controversial whether AICAR-induced AMPK activation is dependent on LKB1 or not (Shaw et al., 2004; Rattan et al., 2005). Although HeLa cell line was known to be deficient of LKB1 expression due to complete methylation at its promoter region (Tiainen et al., 1999), AICAR still could activate AMPK in HeLa cells, which suggests that AICAR-induced AMPK activation is not affected by the absence of LKB1. To confirm this, we tested AMPK activation in MCF7 cells that express LKB1 mRNA normally. AICAR and 8-Cl-cAMP could induce AMPK and p38 MAPK activation in MCF7 cells, just like the results observed in HeLa cells (data not shown). These data suggest that 8-Cl-cAMP and AICAR activate AMPK–p38 MAPK signaling pathway regardless of the presence or absence of LKB1. It also suggests that other AMPKKs other than LKB1 might be in operation after AICAR or 8-Cl-cAMP stimulation in HeLa cells. CaMKKs as suggested can be the candidate for AMPKKs (Hurley et al., 2005) after 8-Cl-cAMP incubation.

In this report, we presented that AMPK and p38 MAPK play important roles in a signaling cascade of 8-Cl-cAMP-induced cell growth inhibition and apoptosis. 8-Cl-cAMP activated AMPK through metabolic conversion and this AMPK activation resulted in the growth inhibition and/or apoptosis, by acting upstream of p38 MAPK. Our findings elucidate the cellular action mechanisms of 8-Cl-cAMP, and contribute to the experimental basis for the therapeutic application of 8-Cl-cAMP or other cancer drugs.

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