

5-Aminoimidazole-4-carboxamide riboside sensitizes TRAIL- and TNF α -induced cytotoxicity in colon cancer cells through AMP-activated protein kinase signaling

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Abstract

Death receptor-mediated tumor cell death, either alone or in combination with other anticancer drugs, is considered as a new strategy for anticancer therapy. In this study, we have investigated the effects and molecular mechanisms of 5-aminoimidazole-4-carboxamide riboside [AICAR; a pharmacologic activator of AMP-activated protein kinase (AMPK)] in sensitizing tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)- and TNF α -induced apoptosis of human colon cancer HCT116 cells. The cytotoxic action of AICAR requires AMPK activation and may occur at various stages of apoptotic pathways. AICAR cotreatment with either TRAIL or TNF α enhances activities of caspase-8, caspase-9, and caspase-3; down-regulates the antiapoptotic protein Bcl-2; increases the cleavage of Bid and results in the decrease of mitochondrial membrane potential; potentiates activation of p38 and c-Jun NH₂-terminal kinase; and inhibits nuclear factor- κ B activity. In addition, this sensitized cell apoptosis was neither observed in p53-null HCT116 cells nor affected by the cotreatment with mevalonate. In summary, we have developed a novel strategy of combining AICAR with TRAIL for the treatment of colon cancer cells. The sensitization effect of AICAR in cell apoptosis was mediated through AMPK pathway, requires p53 activity, and involves mitochondria-dependent apoptotic cascades, p38 and c-Jun NH₂-terminal kinase. [Mol Cancer Ther 2007;6(5):1562–71]

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Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF family, is considered a promising anticancer agent due to its ability to induce apoptosis in a variety of tumor cell types while having only negligible effects on normal cells (1, 2). Sensitivity to TRAIL-induced apoptosis is a key factor influencing the efficacy of TRAIL treatment. Through interaction on death domain-containing receptors [death receptor 4 (DR4) and death receptor 5 (DR5); ref. 3], TRAIL can recruit intracellular adaptor molecule Fas-associated protein with death domain protein (FADD) that engages proximal caspase-8 at the receptor site (4). The activated caspase-8 is released into the cytoplasm and initiates a protease cascade that activates "effector" caspases, such as caspase-3 and caspase-7, thereby triggering the execution steps of apoptosis (extrinsic apoptotic pathway). In addition, active caspase-8 can cleave the proapoptotic Bcl-2 family member Bid generating tBid. tBid in turn translocates to mitochondria and stimulates release of apoptogenic proteins from the mitochondria, thereby amplifying caspase activation (intrinsic apoptotic pathway). In this scenario, the activation of caspase-8, which is considered to be the most apical caspase mediating the extrinsic apoptotic pathway, occurs downstream of effector caspase. Antiapoptotic Bcl-2 family members, such as Bcl-2 and Mcl-1, can support membrane integrity of mitochondria, whereas proapoptotic Bcl-2 family members, such as Bax and Bad, can accelerate intrinsic apoptosis.

AMP-activated protein kinase (AMPK) belongs to a family of serine/threonine protein kinases. The AMPK cascade is a sensor of cellular energy change, which monitors the AMP/ATP ratio to regulate cellular metabolism by restoring ATP levels (5). Several metabolic stresses, including hypoxia, exercise, and long-term starvation, lead to the activation of AMPK (6, 7). 5-Aminoimidazole-4-carboxamide riboside (AICAR) is widely used as a pharmacologic activator of AMPK (5). Although the function of AMPK in metabolism has been well shown, its roles in cell proliferation and cell survival remain poorly documented and somewhat controversial. In agreement with its protective activation from metabolic stresses, AMPK has been proposed as an antiapoptotic molecule to protect cell injury resulting from ischemia (8), hyperglycemia (9), glucose deprivation (10), and ceramide production (11). On the other hand, some reports indicate the ability of AMPK to induce apoptosis of neuroblastoma cells (12, 13), pancreatic cells (14), and gastric cancer cells (15). The latter studies suggest that AMPK signaling might

facilitate growth inhibition and cell killing, serving as a new therapeutic target in cancer diseases (16). Until now, the molecular mechanisms for AMPK-dependent cell arrest and apoptosis remain unclear but might be mediated by up-regulation of p53 and/or inhibition of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase (16). It is likely that the controversial effects of AMPK on cell protection or growth inhibition depend on cell types, cellular events following external stimuli, duration of AMPK activation, and/or downstream-regulated pathways of AMPK.

Given that TRAIL is a promising antitumor agent with no apparent systemic toxicity (17) and that tumor resistance to TRAIL-induced cytotoxicity remains an issue to be solved, recent studies have focused on sensitizing strategy for therapeutic intervention of TRAIL (18–22). Under this context, we wonder whether AMPK activation can have potential application in TRAIL-mediated cancer therapy. Although TNF α cannot be applied in cancer therapy due to marked inflammatory side effects (23), its ability to induce cell death also provides a model to delineate the intracellular apoptotic machinery. Herein, in this study, we concomitantly used TRAIL and TNF α as cytotoxic inducers to elucidate their combinatorial effects with AICAR on cancer cell viability. Moreover, we explored the molecular mechanisms leading to their interacting outcome. Consequently, we observed that the potentiation of TRAIL- and TNF α -induced tumor cell apoptosis by AICAR was dependent on AMPK, p53, involving various aspects of apoptotic pathways, which included the enhancement of Bid degradation, inhibition of Bcl-2 and nuclear factor- κ B (NF- κ B), loss of mitochondrial membrane potential, and activation of c-Jun NH₂-terminal kinase (JNK), p38, and caspases.

Materials and Methods

Reagents

RPMI 1640, fetal bovine serum, penicillin, and streptomycin were obtained from Life Technologies. Polyclonal antibodies against extracellular signal-regulated kinase (ERK) 2, p38 mitogen-activated protein kinase (MAPK), JNK1, p65, p50, Mcl-1_{L/S} (L form and S form), Bcl-2, FADD-like interleukin-1 beta-converting enzyme-like inhibitory protein (FLIP), Bad, Bax, caspase-3, and horseradish peroxidase-coupled antibodies were purchased from Santa Cruz Biotechnology. Bid, caspase-8, caspase-9, AMPK α , poly(ADP-ribose) polymerase, and phosphorylated antibodies of AMPK α , ERK, acetyl-CoA carboxylase α , JNK, ERK, and p38 MAPK were purchased from Cell Signaling. Decoy receptor 2 (DcR2) antibody was purchased from Stressgen. Decoy receptor 1 (DcR1) antibody was purchased from BD PharMingen Technical. U0126, SB203580, and AICAR were purchased from Calbiochem. SP600125 was obtained from Tocris Cookson. Rhodamine-6G was from Sigma. The sequence used to detect the DNA-binding activities of NF- κ B was 5'-GATCAGTTGAGGGGACTTCCCCAGGC-3'. Soluble re-

combinant human TRAIL was purchased from PeproTech and recombinant TNF α , z-Val-Ala-Asp-fluoromethylketone (ZVAD-FMK), and Z-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethylketone (Z-IETD-FMK) were purchased from R&D Systems.

Cell Culture

Human colon adenocarcinoma HCT116 cells were obtained from the American Type Culture Collection. HCT116 p53^{-/-} cells were kindly provided from Dr. Bert Vogelstein (Howard Hughes Medical Institute and The Sidney Kimmel Comprehensive Cancer Center, The Johns Hopkins Medical Institutions, Baltimore, MD). The culture medium used was RPMI 1640, containing 10% fetal bovine serum, 20 mmol/L HEPES buffer, and 100 μ g/mL gentamicin. Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air.

Measurement of Cell Viability by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells (2×10^4 /mL) plated in 96-well plates were incubated with indicated drugs at 37°C for 24 h. MTT (5 mg/mL) was added for 60 min, then the culture medium was removed, and the formazan granules generated by live cells were dissolved in 100% DMSO and shaken for 10 min. The absorbance at 550 and 630 nm was measured using a microplate reader. The net absorbance ($A_{550} - A_{630}$) indicates the enzymatic activity of mitochondria and implicates the cell viability.

Cell Cycle Analysis by Flow Cytometry

Adherent cells after drug treatment were harvested by trypsinization, combined with nonadherent cells, washed twice with PBS, and then fixed with ice-cold 75% ethanol. Cells were resuspended in PBS, and propidium iodide was added and incubated in the dark for 30 min. The samples were analyzed with flow cytometry (Becton Dickinson FACScan). The sub-G₁ peak was quantified and represented the nonviable cell population.

Assessment of Mitochondrial Membrane Potential

Mitochondrial membrane potential ($\Delta\psi_m$) was measured by flow cytometry using the rhodamine-123, whose accumulation in the mitochondria of living cells depends on $\Delta\psi_m$. For doing this, cells were resuspended in PBS containing 10 μ g/mL rhodamine-123 for 15 min at 37°C and then immediately submitted for flow analysis.

Cell Death and Caspase Activity Assay

Cell apoptosis was detected by Cell Death Detection ELISA Plus kit (Roche Diagnostics). Caspase-8 and caspase-3 activities were determined by Colorimetric Assay kits (BioVision). Cell lysates prepared were incubated at 37°C for 2 h. Thereafter, the absorbance at 405 nm was measured with a spectrophotometer.

Transient Transfection and NF- κ B-Dependent Luciferase Assay

Reporter gene containing NF- κ B binding sites (pGL2-ELMA-luciferase) and β -galactosidase expression vector

(pCR3lacZ) were prepared using endotoxin-free plasmid preparation kits (Qiagen). Cells were seeded into 12-well plates overnight before transfection with 0.25 μ g of each plasmid by using LipofectAMINE Plus reagent (Invitrogen).

Immunoblotting Analysis

Cell lysates were harvested as described previously (24), and equal amounts of the soluble protein, as determined by the Bradford protein assay, were denatured, subjected to SDS-PAGE, and transferred to nitrocellulose membrane. Specific protein was then detected by antibody followed by chemiluminescence detection reagent (Amersham Biosciences).

Electrophoretic Mobility Shift Assays

An electrophoretic mobility shift assay reaction mixture contains 100 mmol/L KCl, 5 μ g nuclear protein, 1 μ g poly(deoxyinosinic-deoxycytidylic acid), and 10 fmol γ -³²P-radiolabeled probe for NF- κ B (5'-GATCAGTTGAGGGGACTTCCAGGC-3'). In some samples, specific antibodies against p50 and p65 were cotreated to distinguish the action specificity. After incubation for 20 min on ice, reaction mixtures were resolved by 5% native polyacrylamide gel in TBE buffer.

RNA Interference for AMPK

AMPK α 1 catalytic subunit disruption was achieved by RNA interference using a vector-based small hairpin RNA approach. The target sequence obtained from Dharmacon

RNA Technologies was 5'-UUAAGGCUUCAUCAUCAAUCAUGGU-3' (order number, 1047752; primer number, PRKAA1-HSS108456). In a control group, small interfering RNA with low guanine cytosine content comparable with that of the functional small interfering RNA but lacking specificity for known gene targets was used. Cells were transfected with small interfering RNAs by using LipofectAMINE 2000 (Invitrogen) following the manufacturer's instruction.

Statistical Evaluation

Values were expressed as the mean \pm SE of at least three experiments, which was done in duplicate. ANOVA was used to assess the statistical significance of the differences, and a *P* value of <0.05 was considered statistically significant.

Results

AICAR Enhances the Apoptotic Effects of TRAIL and TNF α

As shown in Fig. 1A, cell viability determined by MTT assay was reduced by TRAIL (50, 100, and 200 ng/mL) and TNF α (50 and 100 ng/mL). After 24-h treatment with 200 ng/mL TRAIL and 100 ng/mL TNF α , cell survival was found to be \sim 70%. However, addition of 1 mmol/L AICAR dramatically enhanced TRAIL and TNF α -induced cell death despite the fact that AICAR alone did not cause

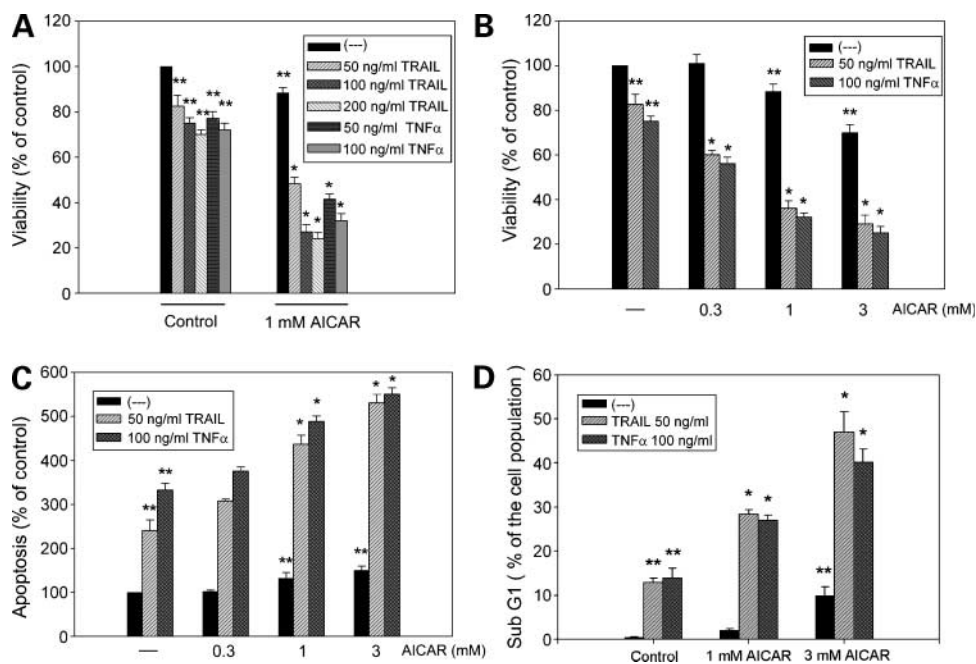
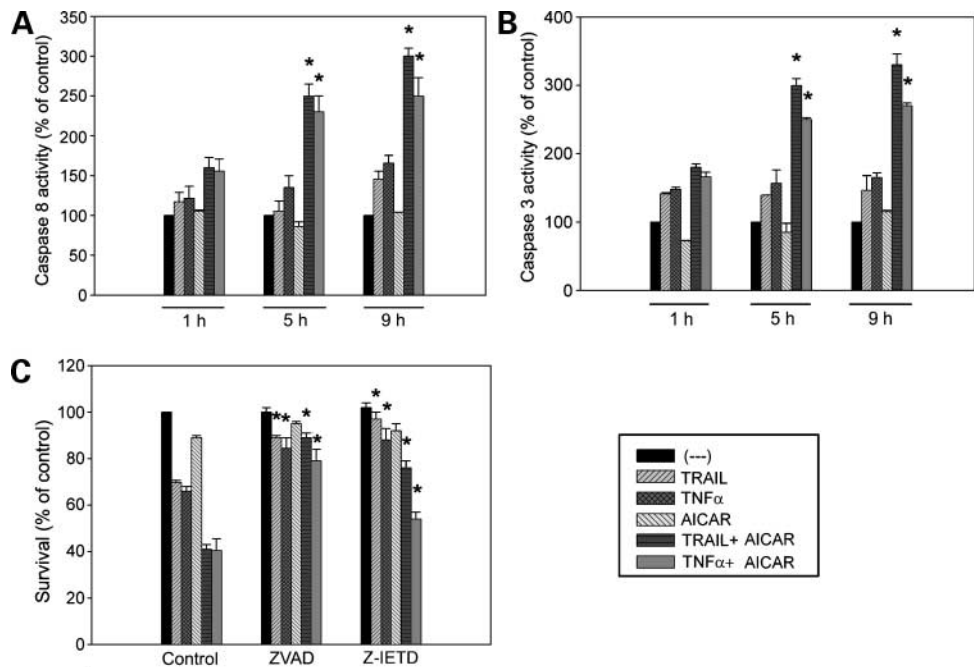


Figure 1. AICAR potentiates the apoptotic effects of TRAIL and TNF α . **A**, HCT116 colon cancer cells were plated in 96 wells, each well contains 2×10^4 cells and treated with TRAIL or TNF α at the indicated concentrations, with or without AICAR (1 mmol/L) for 24 h, and then we used MTT to assay the viability of HCT116. **B**, after treating AICAR at different concentrations (0.3, 1, and 3 mmol/L) with TRAIL (50 ng/mL) or TNF α (100 ng/mL) for 24 h, we measured the survival rate by MTT. **C**, as treated as (**B**), we used Cell Death Detection assay kits to determine the apoptosis level of cells treated with AICAR alone or in combination with cytokines. **D**, HCT116 cells were treated with cytokine alone or in combination with AICAR for 12 h. After overnight fixation, we used flow cytometry to determine the sub-G₁ peak level. Columns, mean of at least three independent experiments; bars, SE. *, *P* < 0.05, indicating the significant potentiation of cell response to cytokines by AICAR; **, *P* < 0.05, indicating significant effects of cytokine and AICAR alone compared with cells without drug treatment.

Figure 2. AICAR enhances caspase activation by TRAIL and TNF α . Cells were treated with agents as indicated for different time intervals (1, 5, or 9 h), and then cell lysates were collected to determine the caspase-8 (A) and caspase-3 (B) activities. C, cells were pretreated with 30 μ mol/L pan-caspase inhibitor [ZVAD-FMK (ZVAD)] or 30 μ mol/L caspase-8 inhibitor [Z-IETD-FMK (Z-IETD)] for 1 h followed by treating TRAIL (50 ng/mL), TNF α (100 ng/mL), AICAR (1 mmol/L), or in combination of these agents for 24 h. The survival was determined by MTT assay. Columns, mean of at least three independent experiments; bars, SE. *, $P < 0.05$, indicating the significant potentiation of cytokine-induced caspase activation by AICAR (A and B) or significant inhibition of cytokine-induced cell death by caspase inhibitors (C).



significant cytotoxicity. Figure 1B showed that AICAR possessed the dose-dependent effects to sensitize cytotoxicity in response to both cytokines after 24-h treatment. Moreover, we used apoptosis assay kits to confirm the dose-dependent effect of AICAR (Fig. 1C). Apoptosis assay was more sensitive than MTT assay, as we observed a slight induction of cell apoptosis by AICAR alone at 1 and 3 mmol/L. Besides using MTT and apoptosis assays, we also did propidium iodide staining to analyze the percentage of cells containing fragmented DNA. As shown in Fig. 1D, treatment of AICAR, TRAIL, or TNF α alone slightly increased sub-G₁ cell fractions; however, AICAR (1 and 3 mmol/L) cotreatment with TRAIL (50 ng/mL) or TNF α (100 ng/mL) for 12 h markedly increased the accumulation of cells in the sub-G₁ phase of the cell cycle.

Because caspase activation is usually a feature of cell apoptosis, using colorimetric caspase assay kits, we found that 1 mmol/L AICAR can potentiate TRAIL and TNF α to induce caspase-8 and caspase-3 activities in different time intervals (5 and 9 h; Fig. 2A and B). To confirm caspase activity contributing to cell death, we pretreated cells with pan-caspase inhibitor (ZVAD-FMK, 30 μ mol/L) or selective caspase-8 inhibitor (Z-IETD-FMK, 30 μ mol/L). As shown in Fig. 2C, significant cell death induced by cytokines, either in the absence or in the presence of AICAR, was reduced by ZVAD-FMK and Z-IETD-FMK. These data indicated that the sensitization of TRAIL- and TNF α -mediated cell death by AICAR depends on caspase activity.

AICAR and Cytokines Synergistically Regulate Mitochondrial Apoptotic Pathways Independent of the Changes of Receptor Expression

Previous studies showed that increased expression of TRAIL death receptor contributes to induction of apoptosis

by chemotherapeutic agents (18, 19, 22). In addition to DR4 and DR5, TRAIL also binds to decoy receptors DcR1 and DcR2, which contain no cytoplasmic death domain and a truncated death domain, respectively. Through competing with DR4 and DR5 for ligand binding, DcR1 and DcR2 act as antagonists. Thus, we examined whether sensitized cell toxicity by AICAR was associated with the changes of TRAIL and TNF α receptor levels. Results revealed that AICAR treatment marginally affected the total cellular levels of DR4, DR5, DcR1, DcR2, and TNFR1 (data not shown). Moreover, quantitative PCR analysis did not reveal the effect of AICAR in mRNA levels of these receptors (data not shown).

Next apoptotic events in mitochondria were evaluated by measuring the mitochondrial membrane potential. As shown in Fig. 3A, we observed that AICAR alone within 24-h incubation did not significantly decrease mitochondrial membrane potential. However, the loss of mitochondrial membrane potential was induced by TRAIL in a time-dependent manner, which achieved 50% loss after 24-h treatment. When cotreated with AICAR, it was further decreased to 30%. Compared with TRAIL alone, TNF α slightly decreased mitochondrial membrane potential by 10% after 24-h treatment, but addition of AICAR synergistically caused 50% potential loss.

We also analyzed by immunoblotting the proteolytic processing of procaspase-8 (upstream initiator caspase), procaspase-3 (downstream effector caspase), procaspase-9, and caspase-3 substrate poly(ADP-ribose) polymerase (Fig. 3B). Although treatment with TRAIL alone resulted in activation of caspase-8 (as indicated by the decrease of full-length procaspase-8 57 kDa and appearance of the cleaved intermediates p43/p41 and active subunit

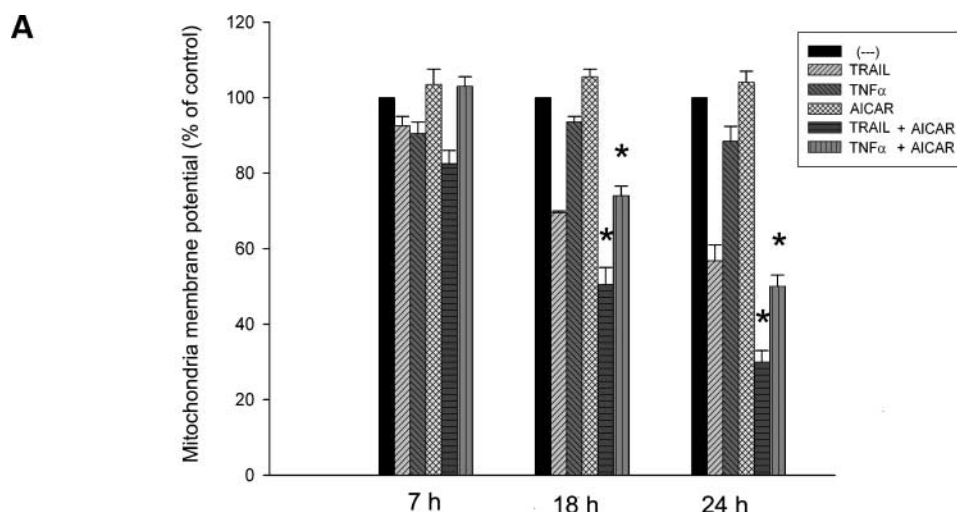
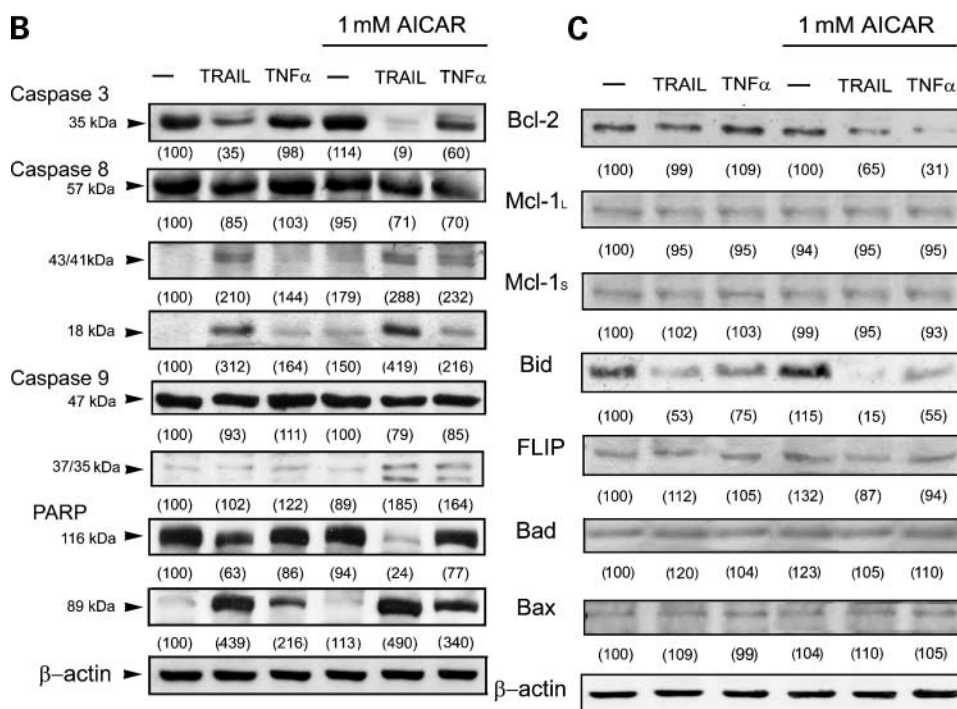


Figure 3. Effects of AICAR on TRAIL- and TNF α -induced apoptotic pathways. **A**, following treatment with TRAIL (50 ng/mL), TNF α (100 ng/mL), and/or AICAR (1 mmol/L) for 7, 18, or 24 h, cells were harvested to measure mitochondrial membrane potential. *Columns*, mean of at least three independent experiments; *bars*, SE. *, $P < 0.05$, indicating the significant potentiation of cytokine-induced response by AICAR. **B** and **C**, cell lysates prepared from cells with similar stimulations as in **(A)** for 8 h **(B)** or 12 h **(C)** were used for immunoblotting with specific antibody. Protein expression levels were determined by densitometry, normalized to the level of β -actin, and expressed as percentages of control without cytokines and AICAR treatment. Traces are representative of three separate experiments.



p18), caspase-3 (indicated by the decrease of procaspase-3 level), and cleavage of poly(ADP-ribose) polymerase (as indicated by the decrease of full-length PARP 116 kDa and production of cleaved fragment 89 kDa), the proteolysis of procaspase-9 (as indicated by the decrease of procaspase-9 and appearance of the cleaved active fragments 37/35 kDa) was less apparent. However, in the presence of AICAR, proteolysis of all these proteins was significantly enhanced. Such action of synergism was also observed in cells cotreated with TNF α and AICAR.

Because several apoptotic molecules are involved in mitochondrial integrity and effector caspase activation, we

postulated that AICAR promoted cell apoptosis by altering other apoptosis-regulating proteins. As shown in Fig. 3C, AICAR costimulation with cytokines leads to the apparent reduction of Bcl-2 and Bid (proform). Nevertheless, levels of Mcl-1_{L/S}, FLIP, Bad, and Bax showed no change in combined treatment.

Roles of MAPKs in AICAR Enhancement of Cell Susceptibility to TRAIL and TNF α

Because MAPKs play important roles in modulation of apoptosis, we determine if such signaling pathways involve in our study. Consequently, we observed that TRAIL, TNF α , and AICAR alone slightly increased the phosphorylated forms of ERK, JNK, and p38 (Fig. 4A–C).

However, the combination of each cytokine and AICAR resulted in synergistic activation of JNK and p38. Increased ERK activation was less prominent than JNK and p38. Next, we found that SB203580 (a selective inhibitor of p38 MAPK) and SP600125 (a selective inhibitor of JNK) can reverse the enhanced cytokine toxicity induced by AICAR, whereas U0126 (a selective inhibitor of MEK) did not show such activity (Fig. 4D).

AICAR Inhibits TRAIL- and TNF α -Induced NF- κ B Activation

Another potential mechanism whereby AICAR regulates TRAIL- and TNF α -induced apoptosis was through interaction with the NF- κ B/Rel family of transcription factor, whose activity has been reported to prevent apoptosis induced by members of the TNF α family. To test this notion, we first determined NF- κ B activity by reporter assay. Figure 5A showed that on cotreating AICAR, which itself had no effect, the increased NF- κ B luciferase activities induced by TRAIL and TNF α were almost abrogated. When we harvested nuclear extract to measure the levels of p65, we detected the ability of AICAR to reduce cytokine-induced nuclear translocation of p65 at 1- and 2-h incubation (Fig. 5B). Moreover, DNA-binding activity as examined by electrophoretic mobility shift assay further showed the ability of AICAR to inhibit TRAIL- and TNF α -induced NF- κ B activation (Fig. 5C).

AMPK and p53 Involve in AICAR-Enhanced Apoptosis

To investigate if AICAR-sensitized apoptosis was mediated through AMPK activation, we first tested whether AICAR can increase the phosphorylated forms of AMPK (an essential step for AMPK activation) and its target substrate (acetyl-CoA carboxylase) in HCT116 cells. Figure 6A revealed the effects of AICAR for inducing AMPK and acetyl-CoA carboxylase phosphorylation. Then, we used small interfering RNA approach specific for AMPK α 1 to abolish the function of AMPK. AMPK α 1 is the major isoform of AMPK α catalytic subunit in HCT116 cells. Under such treatment for 24 h, the synthesis of AMPK α 1 was abolished. Moreover, the subsequent events for AICAR-increased cell apoptosis (Fig. 6B) and caspase-3 activity (Fig. 6C) in response to TRAIL and TNF α were prevented compared with control group.

To determine if p53 might contribute to AMPK-dependent cell apoptosis, we did experiments in p53-null cells. Moreover, as AMPK has been reported to inhibit HMG-CoA reductase, which in turn might contribute to antitumor activity, we addressed this possibility by addition of mevalonate, the direct product of HMG-CoA reductase. As depicted in Fig. 6D, AICAR failed to induce any effect in p53-null cells compared with the sensitized cell death observed in wild-type

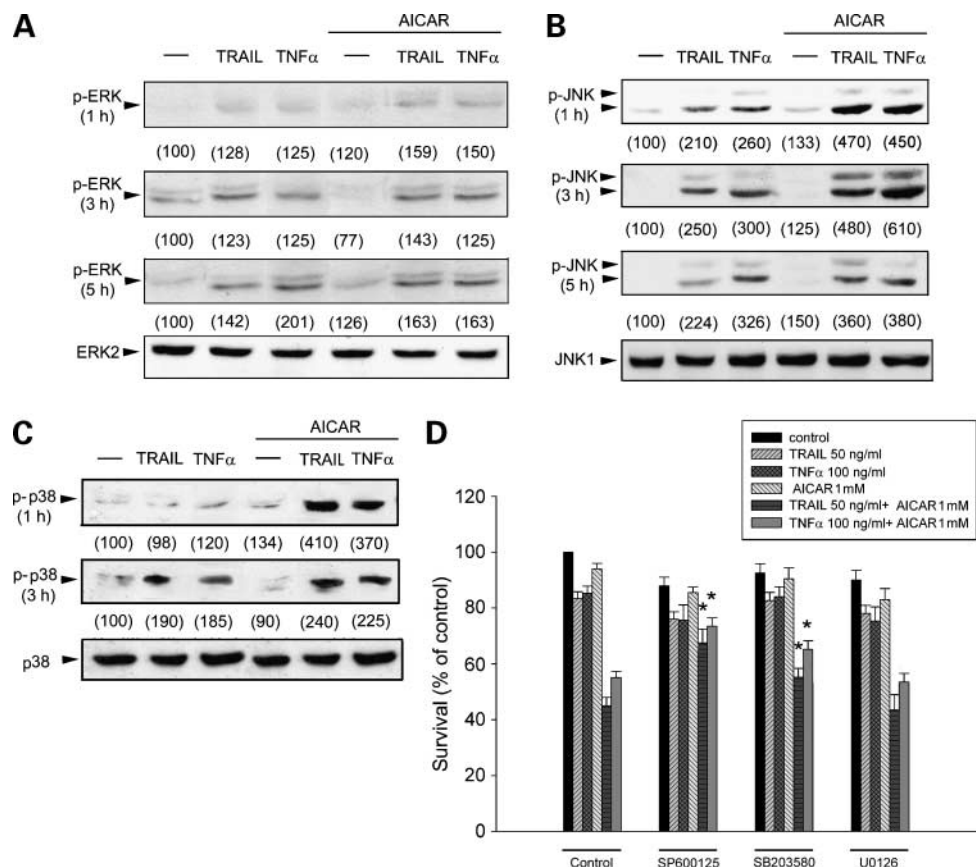


Figure 4. AICAR potentiates TRAIL-activated p38 and JNK. Following stimulations (1 mmol/L AICAR, 50 ng/mL TRAIL, and 100 ng/mL TNF α) for indicated time intervals, cell lysates were used for immunoblotting with antibody specific for phosphorylation or total form of ERK (A), JNK (B), or p38 MAPK (C). In (D), we pretreated cells with SP600125 (10 μ mol/L), SB203580 (10 μ mol/L), or U0126 (10 μ mol/L) for 1 h and then treated with agents as indicated for 24 h. The cell survival was determined by MTT. Columns, mean of at least three independent experiments; bars, SE. *, $P < 0.05$, indicating the significant reverse of cytokine- plus AICAR-induced cell apoptosis by JNK and p38 inhibitors.

HCT116 cells. However, the presence of mevalonate in WT cells did not affect the sensitized cell death caused by AICAR.

Discussion

TRAIL is selectively cytotoxic in inducing tumor cell death and has minimal or no toxicity against normal tissues (17). Thus, TRAIL is a new target with great potential for anticancer treatment, especially in combination with other anticancer therapy. Compared with tumor specificity of TRAIL, the diverse and potent biological actions resulting in inflammation hinder the application of TNF α in tumor therapy (23). Generally, the apoptotic signaling pathways for both death ligands are in common, except the major difference that TRAIL death receptors directly interact with FADD, whereas TRADD is prerequisite to link TNFR1 signaling complex to formation of the FADD-caspase-8 proapoptotic complex. In addition, recruitment of adaptor protein, TNF receptor-associated factor 2, was implicated in TNFR1-induced activation of the antiapoptotic NF- κ B pathway, whereas TNF receptor-associated factor 2 was only modestly recruited to TRAIL death receptors. Although no therapeutic intervention, the present study on the combined effect of AICAR and TNF α will provide a useful model system in mechanistic characterization.

Several recent reports showed that AICAR exhibited both antiapoptotic and proapoptotic effects in different cell types (12, 15, 25). Moreover, AICAR was also shown to suppress cell proliferation (16, 26). In our present study, AICAR reduced HCT116 cell viability significantly and in a dose-dependent manner after treating with TRAIL and TNF α . Here, we used different methods to confirm loss of cell viability, such as MTT assay, apoptosis kits, and measurement of sub-G₁ content. For mechanistic study, we chose 1 mmol/L AICAR to investigate its sensitization effects on TRAIL and TNF α . At this concentration, neither dramatic cell viability nor G₁ cell cycle arrest (data not shown) was caused by AICAR. Previous studies suggest that agents that can arrest cells in the G₀-G₁ phase may increase TRAIL-induced cytotoxicity (27). Our present data further showed that the potentiation of TRAIL- and TNF α -induced apoptosis by AICAR was mediated by AMPK, as we found that this action of AICAR was diminished in cells under si-AMPK α 1 treatment. AMPK α 1 is the predominant catalytic subunit of AMPK α in HCT116 cells. Moreover, we also ruled out the fact that the sensitization effect of AICAR was associated with the changes in the expression of death receptors and decoy receptors.

Caspase- and mitochondria-mediated apoptosis coordinately occur in response to a wide range of death stimuli. Both caspase-8-mediated extrinsic apoptotic pathway and tBid-mediated intrinsic apoptotic pathway can contribute in a widely varying degree to death receptor-induced apoptosis. According to the contribution of caspase-8 and tBid stimulation, two response phenotypes of death receptor-induced apoptosis have been defined. In type I

cells, caspase-8 is robustly activated and by itself is sufficient to trigger effector caspases to an extent allowing the execution of apoptosis. In contrast, in type II cells, death receptor-induced caspase-8 activation alone is not sufficient

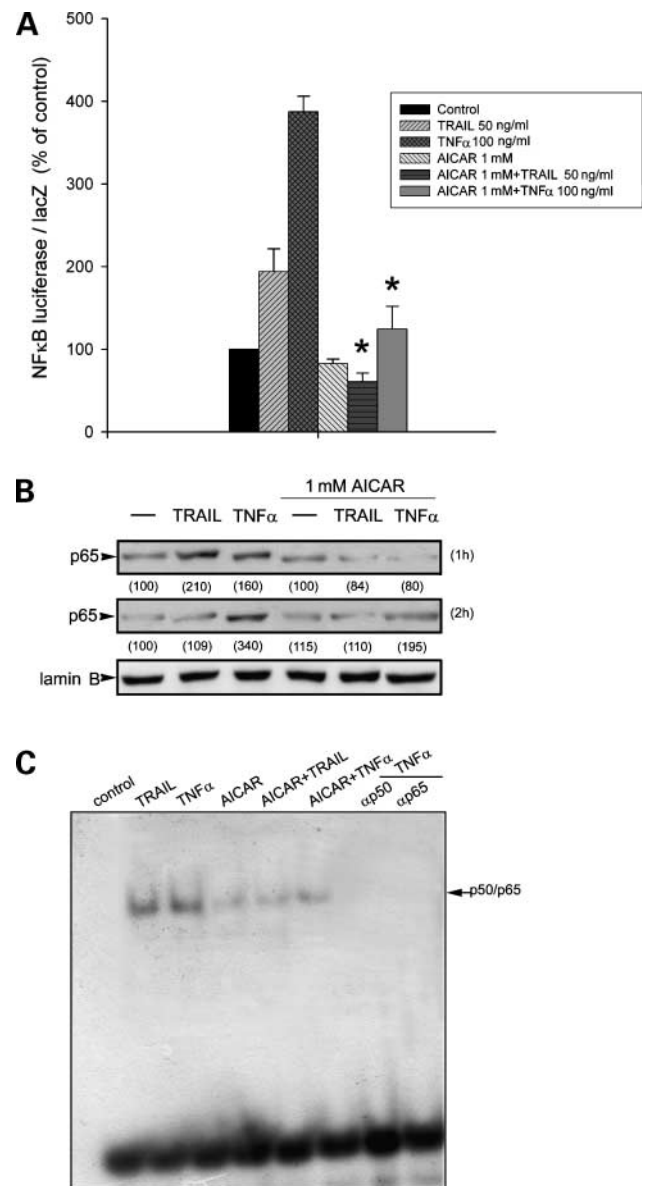


Figure 5. AICAR attenuates NF- κ B activities by TRAIL and TNF α . **A**, HCT116 cells transfected with pGL2-ELMA-luciferase (κ B-Luc) and LacZ plasmids were treated with drugs for 12 h. We used reporter assays to assess the transcription activities of NF- κ B. **Columns**, mean of at least three independent experiments; **bars**, SE. **B**, cells were treated with AICAR (1 mmol/L), TRAIL (50 ng/mL), or TNF α (100 ng/mL) for indicated periods (1 or 2 h), and nuclear fractions of cell lysates were then prepared to conduct immunoblotting for p65. The amount of lamin B was also measured as an internal control. **C**, DNA-binding activity of NF- κ B was examined by electrophoretic mobility shift assay. Cells were treated with AICAR (1 mmol/L), TRAIL (50 ng/mL), and/or TNF α (100 ng/mL) for 30 min, and nuclear fractions of cell lysates were then prepared to conduct electrophoretic mobility shift assay as described in Materials and Methods. Traces are representative of three separate experiments.

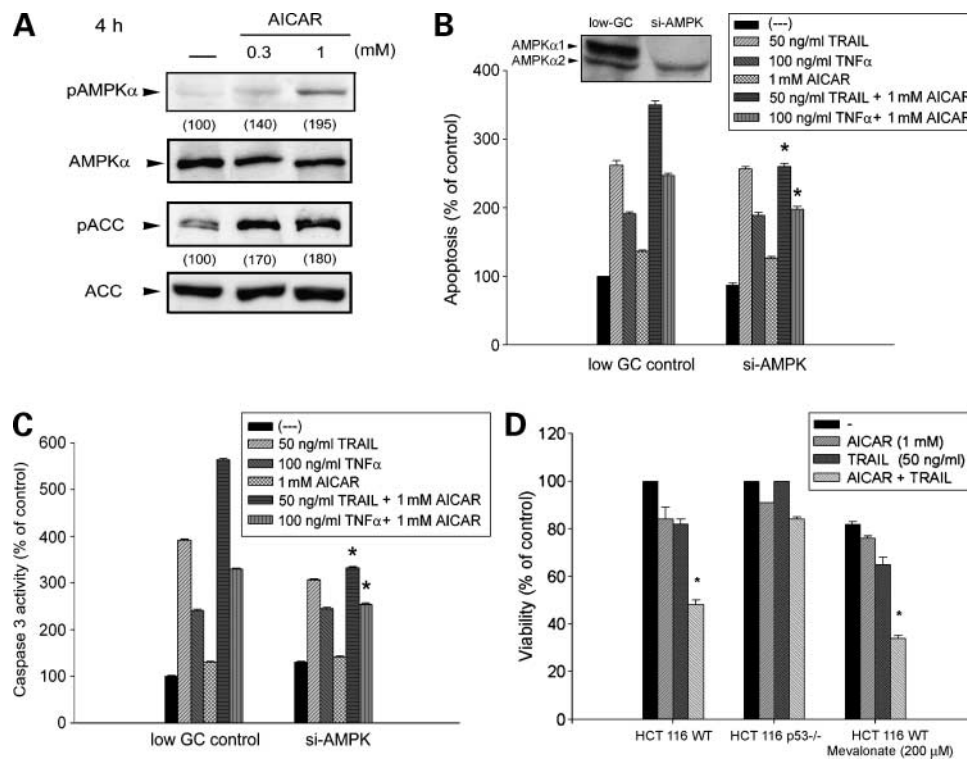


Figure 6. AICAR enhances cytokine-induced cell apoptosis through AMPK- and p53-dependent pathways. **A**, cell lysates prepared from cells with AICAR (0.3 and 1 mmol/L) stimulation for 4 h were immunoblotted with antibody specific for phosphorylated ($pAMPK\alpha$) or total form of AMPK α and phosphorylated ($pACC$) or total form of acetyl-CoA carboxylase. Traces are representative of three separate experiments. **B** and **C**, Cells were transfected with specific AMPK α 1 small interfering RNA to disturb AMPK function or low guanine cytosine (GC) as described in Material and Methods. Cells were stimulated with agents as indicated for 8 h, and then apoptosis was measured by Cell Death Detection Assay kit (**B**) and caspase-3 assay kit (**C**). The transfection efficiency of si-AMPK α 1 was verified by immunoblotting (**B**). In (**D**), wild-type and p53-null HCT116 cells were treated with AICAR (1 mmol/L) and/or TRAIL (50 ng/mL) for 24 h. Cell viability was determined by MTT assay. In some experiments, wild-type cells were cotreated with mevalonate (200 μ mol/L). *Columns*, mean of at least three independent experiments; *bars*, SE. *, $P < 0.05$, indicating the significant inhibition of AICAR potentiation of cytokine-induced cell apoptosis (**B**), caspase activation (**C**), or significant enhancement of TRAIL-induced cytotoxicity by AICAR in wild-type cells (**D**).

to activate effector caspases, and under this condition, tBid-initiated release of apoptogenic mitochondrial proteins can make a significant contribution to apoptosis. In this study, the abilities of TRAIL and TNF α in inducing Bid cleavage, indicating the participation of mitochondria-dependent apoptotic machinery in cell death, led to define HCT116 cells as type II cells. In agreement with our current findings, a previous report also corroborated that HCT116 cells behaved as type II cells in response to TRAIL (28).

We have also shown that sensitization of TRAIL- and TNF α -induced cell death by AICAR was preceded by the enhanced Bid down-regulation, loss of mitochondrial membrane potential, caspase-3, caspase-8, and caspase-9 cleavage and caspase activation. In addition, we also have shown that cell death was prevented by inhibitors of caspase-8 (Z-IETD-FMK) and pan-caspase inhibitor (ZVAD-FMK). These results suggested that mitochondrial involvement in AICAR-induced tumor cell susceptibility to TRAIL and TNF α and that the apoptotic pathways triggered by cytokine alone or in combination with AICAR were converged to caspase activation. Here, we also found that compared with TRAIL, the TNF α -activated loss of

mitochondrial membrane potential and Bid cleavage were less apparent. These findings suggested that under the tested concentrations of cytokines, intrinsic apoptotic pathway played a minor role in HCT116 cells treated with TNF α compared with TRAIL. One possible mechanism for such differential response might be ascribed to the more prominent effect of TNF α on NF- κ B activation than that of TRAIL.

Bcl-2 family was well recognized to regulate intrinsic apoptosis pathway, and some antitumor drugs can induce apoptosis in cancer cells by modulating Bcl-2 protein family. As reported, the antiapoptotic molecules Bcl-2 and FLIP can protect some tumor cell lines from TRAIL-induced apoptosis (29, 30), and proapoptotic molecule Bax was essential for the apoptosis of HCT116 cells after treatment with TRAIL (31). Under these contexts, both antiapoptotic molecules (such as FLIP, Bcl-2, and Mcl-1) and proapoptotic molecules (such as Bid, Bad, and Bax) were examined in this study. Among them, we showed that the presence of AICAR and cytokines could down-regulate Bcl-2 while up-regulating Bid cleavage. Down-regulation of the antiapoptotic Bcl-2 may increase the available concentrations of tBid, leading to a greater release of apoptotic

proteins from the mitochondria. Activation of p53, a key step for inducing intrinsic apoptosis (32), has been identified to sensitize cancer cell response of TRAIL (33). Because one proposed mechanism for AMPK-mediated antiproliferation was mediated by p53 (16), we examined the role of p53 in AICAR-mediated cell death. Based on results obtained from p53-null HCT116 cells, we showed that AMPK-mediated intrinsic apoptotic sensitization requires p53 activation.

Despite the paucity of information, inhibition of HMG-CoA reductase might contribute to antitumor activity (34). In this aspect, HMG-CoA reductase inhibitors (statins) were shown to increase TRAIL-induced cytotoxicity (27). Proposed antitumor mechanisms of HMG-CoA reductase inhibition were primarily related to reduced levels of mevalonate and its downstream products (including geranylpyrophosphate and farnesylpyrophosphate), many of which play important roles in cell proliferation and survival (34). AMPK activation has been reported to phosphorylate and inhibit this enzyme and reduce cholesterol levels (16). Here, we showed that cell death-promoting effect of AICAR might not be mediated through the inhibition of HMG-CoA reductase. We have observed that exogenous mevalonate did not overcome the death-inducing effect of AICAR, although it can prevent biological outcome of statins (24, 35).

Contribution of MAPKs in cell apoptosis has been extensively documented. As indicated previously, p38 MAPK and JNK activation lead to mitochondrial apoptotic pathway (36, 37), and JNK activation promotes processing of Bid to proapoptotic cleavage product (38). To date, several studies using pharmacologic or molecular approaches have indicated that AMPK was an upstream regulating molecule for activation of p38 (37, 39, 40), JNK (14, 41), and ERK (42). Our results using selective inhibitors indicated that JNK and p38 contribute to AICAR sensitization of TRAIL- and TNF α -induced apoptosis, and their activities were actually enhanced by the presence of AICAR together with TRAIL and TNF α . We therefore suggest that p38 and JNK may be involved in mitochondrial apoptotic pathway.

NF- κ B is widely known for its ubiquitous roles in cell survival and apoptosis (43). NF- κ B protects cells from apoptosis by transcriptional activation of survival factors, such as c-FLIP, XIAP, c-IAP, A1, Bcl-2, and Bcl-xL. This ability explains the role of NF- κ B as an inhibitor of cell death. Our current data showed that AICAR was able to inhibit NF- κ B activity induced by TRAIL and TNF α . This conclusion was supported by immunoblotting, showing the inhibition of p65 nuclear translocation; electrophoretic mobility shift assay, showing the inhibition of DNA binding; and reporter assay, showing the inhibition of transcriptional activity. This inhibition on NF- κ B by AICAR was consistent with the previous report in glial cells (44).

In summary, we showed that AICAR, through AMPK signaling pathway, sensitizes death receptor-mediated cytotoxicity in human colon cancer cells. Enhancement of mitochondria-dependent apoptotic cascades, concomitant

sensitization of p38 and JNK activities, and inhibition of NF- κ B activity all contributed to the sensitized cytotoxic action of AICAR. This enhanced tumor-directed activity identified AMPK signaling as a potential target for cancer therapeutics, especially in current progress made toward improving the therapeutic efficacy by targeting and activation of death receptors. Another property that might benefit the development of AMPK activator in cancer therapy was the identification of its cytoprotective functions rather than cytotoxicity exerted by AMPK in normal cells that respond to stress.

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