AICAR Induces Apoptosis in Human Osteosarcoma Cells through AMPK-dependent PGC-1α/TFAM/Mitochondrial Pathway

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Introduction: Osteosarcoma is the most common form of primary malignant bone tumor, which has a very high rate of metastasis and the prognosis for patients with metastatic osteosarcoma is generally poor (1, 2). Recently, the relationship between cancer cell growth and cell metabolism has been pointed out, the energy regulating enzyme AMP-activated protein kinase (AMPK) being one of its main players. Activation of AMPK acts to maintain cellular energy stores, switching on catabolic pathways that produce ATP, mostly by enhancing oxidative metabolism and mitochondrial biogenesis, while switching off anabolic pathways that consume ATP (3). 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) is widely used as a pharmacologic activator of AMPK. AICAR was also shown to stimulate mitochondrial biogenesis in skeletal muscle, and has been shown to be an exercise mimetic (4). Previous studies have revealed that AICAR inhibited cell proliferation and induced apoptosis in various cancer cells, including neuroblastoma (5), colon cancer (6), breast cancer and prostate cancer cells (7). However, in osteosarcoma cells, the potential role of AMPK and/or the anticancer effect of AICAR have not been addressed. We previously reported that increasing the mitochondrial numbers through PGC-1alpha overexpression induced mitochondrial apoptosis in human sarcoma cell lines (8). Therefore, we hypothesize that AICAR could increase PGC-1alpha overexpression by AMPK activation, and could induce mitochondrial apoptosis through PGC-1alpha/TFAM/mitochondrial pathway in osteosarcoma cells. The present study was undertaken to evaluate the effect of AICAR on apoptotic activity through AMPK phosphorylation in human osteosarcoma cells in vitro.

Methods: Cells.
Human osteosarcoma cell lines (MG63 and KHOS) were cultured in DMEM containing 10% FBS and 100 U/mL penicillin/streptomycin solution. Cells were routinely maintained at 37°C in a humidified 5% CO2 atmosphere.

Effect of AICAR on AMPK Activation and PGC-1alpha/TFAM Pathway in Osteosarcoma Cells.
In order to test the effect of AICAR on AMPK activation, protein expressions of AMPKalpha and phosphorylated AMPKalpha in AICAR treated osteosarcoma cells were evaluated by immunoblot analysis. To investigate the effect of AICAR on PGC-1alpha/TFAM pathway, total RNA was extracted from cells at 48 hours of AICAR treatment, and mRNA expressions of PGC-1alpha, NRF-1 and TFAM were evaluated by quantitative real time PCR.

Effect of AICAR on Osteosarcoma Cell Growth.
The effect of AICAR on osteosarcoma cell growth was assessed by cell viability assay. Cells were set up 5000 cells of 96-well plates and treated by various concentrations of AICAR (0-2 mM). Cell viability after AICAR treatment was examined using WST Cell Counting Kit (Dojindo, Japan).
Apoptotic Activity After AICAR Treatment in Osteosarcoma Cells.

To evaluate the effect of AICAR on osteosarcoma apoptosis, cell lysates were collected from cells at 72 hours of AICAR treatment, and expressions of apoptosis-related proteins, such as caspase-3, caspase-9 and PARP, were assessed by immunoblot analysis. And, DNA fragmentation was assessed by flow cytometry. Immunofluorescence staining was also performed to verify the relationship between mitochondrial proliferation and cellular apoptosis in AICAR treated osteosarcoma cells using an APO-Direct Kit (BD Pharmagen) and MitoTracker Deep-Red FM (Invitrogen).

Statistical Analysis.

Each experiment was performed independently at least three times. Data are presented as mean ± SE unless otherwise indicated. Statistical significance of differences between mean were evaluated by two-tailed Student’s t-tests. All tests were considered significant at p<0.05.

Results: AICAR Induced AMPK Phosphorylation and Up-regulation of PGC-1alpha/TFAM/Mitochondrial Pathway.

As shown in Fig.1A, the expression of phosphorylated AMPKalpha (Thr 172) was increased immediately after AICAR treatment in MG63 osteosarcoma cells. In quantitative real time PCR, a significant increase in mRNA expressions of PGC-1alpha, NRF1 and TFAM were observed in AICAR treated cells compared with control cells (Fig.1B). The findings strongly suggest that AICAR could induce AMPK activation and up-regulation of PGC-1alpha/TFAM/mitochondrial pathway in osteosarcoma cells.

AICAR Inhibited Cell Growth and Induced Mitochondrial Apoptosis in Osteosarcoma Cells.

Cell viability assay revealed that AICAR dose-dependently inhibited cell viability in both osteosarcoma cell lines at 48 hours of treatment (Fig. 2). Immunoblot analysis showed that the expressions of cleaved forms of caspase-9, caspase-3 and PARP were also strongly increased in AICAR treated osteosarcoma cells at 72 hours of treatment (Fig. 3). Immunofluorescence staining revealed that increased apoptotic cell numbers with mitochondrial proliferation were observed in AICAR treated osteosarcoma cells. These observations suggested that AICAR induced mitochondrial apoptosis through AMPK activation in human osteosarcoma cells.

Discussion: In the present study, we observed that AICAR-induced mitochondrial apoptosis with AMPK phosphorylation and up-regulation of PGC-1alpha/TFAM/mitochondrial pathway in osteosarcoma cells. To the best of our knowledge, this is the first report that revealed apoptotic effect of AICAR through AMPK and mitochondrial pathway in osteosarcoma cells. Fu et al. reported that, in HeLa cervical carcinoma cells, AMPK phosphorylation increased the mRNA levels of PGC-1alpha, NRF-1 and TFAM resulting in up-regulation of mitochondrial DNA replication and transcription (9). And also, we have previously elucidated the relationship between mitochondrial biogenesis and musculoskeletal tumorigenesis (8). We observed in the studies that increasing mitochondrial numbers by PGC-1alpha overexpression induced cellular apoptosis in human sarcoma cell lines and suggest that regulation of the PGC-1alpha/TFAM/mitochondrial pathway may be a potent therapeutic target for human musculoskeletal malignancies (8). Taken together, AMPK phosphorylation by AICAR could induce mitochondrial apoptosis via PGC-1alpha/TFAM pathway, and AICAR might be considered as a potent therapeutic agent for the treatment of osteosarcoma.

Significance: AICAR induces apoptosis in human osteosarcoma cells through AMPK-dependent PGC-1alpha/TFAM/mitochondrial pathway, and AMPK activation by AICAR might be considered as an attractive target for osteosarcoma therapy.
Fig. 1 Effect of AICAR on AMPK phosphorylation and the up-regulation of PGC-1alpha/TFAM/mitochondrial pathway
MG63

IC50 = 369μM

Fig. 2 Effect of AICAR on cell viability
Fig. 1 Effect of AICAR on AMPK phosphorylation and the up-regulation of PGC-1alpha/TFAM/mitochondrial pathway.