Biochemical and Biophysical Research Communications 474 (2016) 277–283

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

AICAR induces AMPK-independent programmed necrosis in prostate cancer cells

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ARTICLE INFO

Article history:
Received 12 April 2016
Accepted 16 April 2016
Available online 18 April 2016

Keywords:
AICAR
Prostate cancer
Programmed necrosis
Cyclophilin-D
AMPK

ABSTRACT

AICAR (5-Aminoimidazole-4-carboxamide riboside or acadesine) is an AMP-activated protein kinase (AMPK) agonist, which induces cytotoxic effect to several cancer cells. Its potential activity in prostate cancer cells and the underlying signaling mechanisms have not been extensively studied. Here, we showed that AICAR primarily induced programmed necrosis, but not apoptosis, in prostate cancer cells (LNCaP, PC-3 and PC-82 lines). AICAR's cytotoxicity to prostate cancer cells was largely attenuated by the necrosis inhibitor necrostatin-1. Mitochondrial protein cyclophilin-D (CYPD) is required for AICAR-induced programmed necrosis. CYPD inhibitors (cyclosporin A and sanglifehrin A) as well as CYPD shRNAs dramatically attenuated AICAR-induced prostate cancer cell necrosis and cytotoxicity. Notably, AICAR-induced cell necrosis appeared independent of AMPK, yet requiring reactive oxygen species (ROS) production. ROS scavengers (N-acetylcysteine and MnTBAP), but not AMPKα shRNAs, largely inhibited prostate cancer cell necrosis and cytotoxicity by AICAR. In summary, the results of the present study demonstrate mechanistic evidences that AMPK-independent programmed necrosis contributes to AICAR's cytotoxicity in prostate cancer cells.

1. Introduction

Prostate cancer is an important cause of men's cancer-associated mortalities in China [1] and around the world [2–4]. In the United States alone, it is estimated that one in every nine men over the age of 65 will be diagnosed with this disease [2]. Over the past decades, significant developments have been achieved in clinical aspects of prostate cancer, including diagnosis, surgery treatment and chemotherapy [5–7]. Yet, for those with recurrent or metastatic prostate cancer, the prognosis has not been improved [5–7]. Therefore, there is a vital need to develop alternative chemotherapeutic strategies against prostate cancer [5–7]. It has also been our research focus in many years [8].

The nucleoside 5-aminoimidazole-4-carboxamide riboside (AICAR) is a low-energy mimetic and adenosine monophosphate (AMP)-activated protein kinase (AMPK) agonist [9]. Recent studies have shown that AICAR could exert cytotoxic effect via AMPK-dependent and/or AMPK-independent mechanisms [10,11]. Its potential roles in prostate cancer cells, and more importantly the underlying mechanisms of its actions have not been extensively studied. Here our results suggest that necrosis could be the major form of cell death-induced by AICAR in prostate cancer cells.

Cell necrosis has long been considered as a passive and uncoordinated type of cell death. Yet, recent studies (including ours [8]) have indicated that necrosis, just like apoptosis, is a molecularly regulated event, which is named as “programmed necrosis” [12–16]. A number of stimuli, including ischemia-reperfusion injury, oxidative stresses, neurodegeneration, and ultraviolet radiation, as well as several anti-cancer agents were reportedly to induce the programmed necrosis pathway [12–16]. Our previous study has shown that berberine-induced cytotoxicity in prostate cancer cells is mainly through inducing the cell necrosis (with only some apoptosis) [8]. Therefore, one major focus of this study is to test the possible involvement of the programmed necrosis pathway in AICAR's cytotoxicity against prostate cancer cells.

Studies have confirmed that mitochondrial permeability transition pore (mPTP), a mitochondrion localized channel complex, plays a central role in mediating programmed necrosis [15,16]. mPTP is primarily composed of voltage-dependent anion channel (VDAC, in the outer membrane), the adenine nucleotide translocator (ANT, in the inner membrane), and cyclophilin D (CYPD, in

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http://dx.doi.org/10.1016/j.bbrc.2016.04.077
0006-291X/

the mitochondrial matrix) [17,18]. Cytotoxic stimuli will promote CYPD association with ANT, which then triggers mPTP opening [19,20]. This will lead to mitochondrial depolarization, mitochondria swelling, and eventually cell necrosis [19,20]. In the present study, we show that AICAR induces CYPD/mPTP-dependent programmed necrosis in prostate cancer cells.

2. Materials and methods

2.1. Chemicals and reagents

AICAR, genistein, N-acetylcysteine (NAC), MnTBAP, sanglifehrin A and cyclosporine A were obtained from Sigma (St. Louis, MO); Z-VAD-fmk and necrostatin-1 [8] were purchase from Calbiochem (Shanghai, China). All antibodies utilized in this study were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cell culture

Human prostate carcinoma PC-3, PC-82 and LNCaP cell lines [8] were cultured in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS, Hyclone, Shanghai, China).

2.3. Cell viability assay

The methyl thiazolyl tetrazolium (MTT, Sigma) assay was performed to assess the cell viability [8]. The value of treatment group was expressed as percentage change of that of untreated control group.

2.4. Trypan blue staining of "dead" cells

Following indicated treatment, the percentage (%) of cell death was calculated by the number of the trypan blue positive cells divided by the total number of the cells.

2.5. Clonogenicity assay of cell proliferation

The prostate cancer cells (5×10^3 per dish) were suspended in 1 mL of DMEM containing 0.25% agar (Sigma), 10% heat-inactive FBS plus applied AICAR treatment. The cell suspension was then added on top of a pre-solidified 100 mm culture dish. The medium was replaced every two days. After 12 days of incubation, the remaining survive colonies were counted manually.

2.6. Lactate dehydrogenase (LDH) assay

LDH content in the conditional medium indicates the level of cell necrosis. After treatment, the LDH content was measured via the LDH detection kit (Biyuntian, Wuxi, China). LDH release % = LDH in conditional medium/(LDH in conditional medium + LDH in cell lysates) x 100%. Cells were lysed by the lysis buffer attached in the kit.

2.7. Annexin V FACS assay of cell apoptosis

Apoptosis was quantitatively determined by flow cytometry using the Annexin V Apoptosis Detection Kit (BD, Shanghai, China) following the manufacturer's instructions. Detailed protocol was described in our previous study [8]. Percentage of Annexin V positive cells was recorded as apoptosis ratio [8].

2.8. Quantification of apoptosis by enzyme-linked immunosorbent assay (ELISA)

The Cell Apoptosis Histone-DNA ELISA Detection Kit (Roche, Palo Alto, CA) was applied to quantify cell apoptosis via the method described in other studies [21,22]. ELISA OD was recorded as a quantitative measurement of cell apoptosis.

2.9. Assay of caspase-3 activity

The prostate cancer cells were seeded onto 96-well plats. Following applied treatment, caspase-Glo reagent (100 μL/well) was added, using the specific caspase-3 substrate DEVD-AFC as the substrate. Caspase-3 activity was determined via the caspase-Glo 3 kit (Promega, Shanghai, China), and was normalized to that of the untreated control group.

2.10. Reactive oxygen species (ROS) detection

Intracellular ROS generation was measured by flow cytometry via dichlorofluorescin (DCF) oxidation assay as described in our previous study [8]. Briefly, after treatment, cells were washed with PBS and were incubated with DCFH-DA (5 μM) for 1 h at 37 °C. Thereafter, ROS fluorescence was analyzed via detecting DCF intensity. The value of treatment group was normalized to that of untreated control group [8].

2.11. Mitochondrial membrane potential (MMP) detection

As described [8], the mitochondrial membrane potential reduction ($\Delta\psi_m$) was determined using the lipophilic cationic probe JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide) (Molecular Probes Inc., Eugene, OR). The MMP reduction ($\Delta\psi_m$) was reflected by the increase of JC-1 green fluorescence intensity. The value of the treatment group was normalized to that of untreated control group [8].

2.12. Western blotting

After treatment, cells were solubilized in the lysis buffer described [8]. For SDS-PAGE, 20 μg of total proteins per sample were loaded to each lane, and transferred to PVDF membranes. Targeted proteins were detected with specific primary antibodies. Corresponding secondary antibodies were utilized, and antibody-antigen binding was visualized by enhanced chemiluminescence (ECL).

2.13. Mitochondrial immunoprecipitation (Mito-IP)

To detect mitochondrial protein, the mitochondrial fraction was isolated via the "Mitochondria Isolation Kit for Cultured Cells" (Pierce, Rockford, IL) [8], mitochondrial lysates (600 μg per sample, from roughly 10 million cells per sample) were pre-cleared with 30 μL of protein IgA/IgG-beads (Sigma). The supernatant was then rotated overnight with 0.2 μg of anti-ANT-1/2 (Santa Cruz) overnight. The protein IgA/IgG-beads (35 μL) were then added again to the supernatant for 12 h at 4 °C. Then, the pellets were washed six times with cold PBS and 1 time with lysis buffer, and then assayed in Western blotting to detect the ANT1/2-CYPD association.

2.14. shRNA

The two sets of lentiviral particles with human CYPD shRNAs were purchased from Santa Cruz Biotech ("CYPD shRNA sequence-a" [8]) and Genechem ("CYPD shRNA sequence-b" [23,24],