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AICAR induces Nrf2 activation by an AMPK-independent mechanism in hepatocarcinoma cells

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ABSTRACT

Hepatocellular carcinoma is one of the most frequent tumor types worldwide and oxidative stress represents a major risk factor in pathogenesis of liver diseases leading to HCC. Nuclear factor erythroid 2-related factor (Nrf2) is a transcription factor activated by oxidative stress that governs the expression of many genes which constitute the antioxidant defenses of the cell. In addition, oxidative stress activates AMP-activated protein kinase (AMPK), which has emerged in recent years as a kinase that controls the redox-state of the cell. Since both AMPK and Nrf2 are involved in redox homeostasis, we investigated whether there was a crosstalk between the both signaling systems in hepatocarcinoma cells. Here, we demonstrated that AMPK activator AICAR, in contrary to the A769662 allosteric activator, induces Nrf2 activation and concomitantly modulates the basal redox state of the hepatocarcinoma cells.

When the expression of Nrf2 is knocked down, AICAR failed to induce its effect on redox state. These data highlight a major role of Nrf2 signaling pathway in mediating the AICAR effect on basal oxidative state. Furthermore, we demonstrated that AICAR metabolization by the cell is required to induce Nrf2 activation while, the silencing of AMPK does not have any effect on Nrf2 activation. This suggests that AICAR-induced Nrf2 activation is independent of AMPK activity. In conclusion, we identified AICAR as a potent modulator of the redox state of human hepatocarcinoma cells, via the Nrf2 signaling pathway and in an AMPK-independent mechanism.

1. Introduction

Primary liver cancer represents the sixth most common cancer and the third leading cause of cancer mortality [1–3]. Hepatocellular carcinoma (HCC), accounts for between 85% and 90% of such liver cancers. Alcohol abuse, chronic viral hepatitis, obesity, autoimmune hepatitis, metabolic disorders, persistent exposure to toxins and chemicals are major risk factors in the pathogenesis of liver diseases leading to HCC [4]. All these chronic stimuli may induce oxidative stress through increased generation of reactive oxygen species (ROS) and a decrease in the antioxidant defense levels such as glutathione (GSH), catalase or superoxide dismutase (SOD) [5,6]. Overall, these events may induce oxidative DNA damage, which in turn increases chromosomal aberrations associated with cell transformation [7,8]. Impairment of lipid peroxidation following oxidative stress is also critically involved in the progression of liver cancer [9].

The transcriptional factor Nuclear factor erythroid 2-related factor (Nrf2) governs the expression of many genes involved in cell protection against oxidative insults and which constitute the

Abbreviations: AMPK, AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide riboside; ARE, antioxidant response element; GSH, reduced glutathione; HCC, hepatocellular carcinoma; Keap1, Kelch-like ECH associating protein 1; Nrf2, nuclear factor erythroid 2-related factor; ROS, reactive oxygen species.

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antioxidant defenses of the cell [10]. Under basal conditions, Nrf2 is bound to Kelch-like ECH associating protein 1 (Keap1) in the cytoplasm [11]. Keap1 acts as substrate linker protein interaction of the Cul3-based E3-ubiquitin ligase complex with Nrf2 leading to ubiquitination of Nrf2 and proteosomal degradation [12,13]. In response to oxidative stress, some critical cysteine residues are oxidized leading to Keap1 conformational changes.

As a consequence, the newly synthesized Nrf2 molecules escape the capture by Keap1 and translocate into the nucleus. By dimerizing with small Maf protein, Nrf2 will bind to the antioxidant response element (ARE) and promote the transcription of the corresponding target genes [14,15] such as glutathione reductase, peroxiredoxin, thioredoxin, catalase, SOD and heme oxygenase 1 (HO-1) [16]. Hence, the antioxidant properties of Nrf2 make it a promising target for the therapy of liver cancer.

Oxidative stress appears to activate AMP-activated protein kinase (AMPK) signaling system, which has emerged in recent years as a kinase that controls the redox-state of the cell [17–19]. AMPK is an evolutionarily conserved serine/threonine kinase that is ubiquitously expressed. It is a heterotrimer consisting of a catalytic α-subunit and two regulatory subunits, β and γ. AMPK was initially characterized as a "fuel gauge", modulating cellular energy flux in eukaryotic cells in response to changes in the AMP/ATP ratio [20]. Indeed, AMPK is activated by conditions that increase intracellular AMP such as hypoglycemia, hypoxia and exercise, as well as certain cytokines and drugs, such as metformin and 5-aminoimidazole-4-carboxamide riboside (AICA riboside or AICAR) [21]. AICAR is a cell-permeable nucleoside which could be metabolically converted to AICA ribotide (or ZMP), the antepenultimate metabolic intermediate of the de novo purine synthesis pathway [22]. The liver kinase B1 (LKB1) and Ca^{2+}/calmodulin-dependent protein kinase kinase-β (CaMKKβ) are upstream kinases that activate AMPK by phosphorylating Thr172 in the activation loop of the catalytic α-subunits [23,24]. It is now becoming clear that metabolic stress is not the only pathological or physiological condition that activates AMPK and that the function of this enzyme extends beyond metabolic control and energy homeostasis, to include the control of redox state. Indeed, increased intracellular concentration of ROS may represent a general mechanism for the enhancement of AMPK-mediated cellular adaptation, including maintenance of redox homeostasis [19].

Recently, AMPK was identified as a novel inducer of HO-1 in human endothelial cells (ECs), via the Nrf2/ARE signaling pathway [25]. In this study, treatment of ECs with the AMPK activator AICAR stimulated an increase in HO-1 expression that was associated with an increase in Nrf2 protein abundance. In addition, activation of AMPK by metformin in *C. elegans* was shown to promote nuclear translocation of SKN-1 (orthologous to the mammalian Nrf2) and expression of its target *gst-4* [26]. Since both AMPK and Nrf2 are activated by oxidative stress and involved in the maintenance of redox homeostasis, we investigated whether AMPK is involved in redox homeostasis regulated by Nrf2, and more precisely whether AMPK could induce the activation of Nrf2 in HepG2 hepatocarcinoma cells. To this end we utilized two well-known AMPK activators, namely AICAR which is converted in cells to the AMP analog ZMP [27,28], and A769662 (Abbott), a non-nucleotide small-molecule, thienopyridone, which is an allosteric activator [29]. Thus, A769662 is the best available AMPK activator, while the prototypic AMPK activator, AICAR, is yet less specific.

2. Materials and methods

2.1. Chemicals

5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) was purchased from Toronto Research Chemicals (North York, ON, Canada). A769662 (Abbott) was kindly given by Dr A. Balandran (AstraZeneca, Mölndal, Sweden). Iodotubercidin (Iodo), tert-butylhydroquinone (tBHQ) and sucrose were from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were ACS reagent grade. Recombinant GST-human Keap1 chimera was from Sino Biological Inc (Interchim, Montlçon, France).

2.2. Cell culture

The human hepatocarcinoma cell line HepG2-E47 was a gift from Dr. A. Cederbaum (Department of Pharmacology and Systems Therapeutics, Mount Sinai School of Medicine, New York, USA) [30]. The cells were cultured as monolayer in MEM-GlutaMAX™-I (Life Technologies, Merelbeke, BE) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and G418 (500 μg/ml). The medium was changed at 48–72 h intervals. All cultures were maintained in a humidified incubator at 37 °C under 5% CO_2. For each passage, cells were washed twice with PBS and then incubated at 37 °C with 0.25% trypsin–EDTA (Sigma–Aldrich).

2.3. Glutathione content

The glutathione content was determined by using the Tietze enzyme recycling assay [31], with slight modifications [32]. Cells were washed twice with ice-cold phosphate buffered saline and then lysed with a solution of 5-sulfosalicylic acid (5%). After two freeze–thaw cycles, samples were centrifuged at 10 000 × *g* for 10 min and the resulting supernatants were kept at −80 °C until used. Ten microliters of the samples were then placed in a reaction mixture containing 0.2 U/ml of glutathione reductase, 50 μg/ml 5,5′-dithiobis(2-nitrobenzoic acid) and 1 mM ethylenediaminetetraacetaticacid (EDTA) at pH 7. The reaction was initiated after adding 50 μM NADPH and changes in absorbance were recorded at 412 nm. Reduced (GSH) and oxidized (GSSG) glutathione were distinguished by the addition of methyl-2-vinylpyridine and their respective concentrations were determined from appropriate standard curves. Results were normalized to the protein content, which was measured using the method of Lowry [33].

2.4. Intracellular ROS determination

Cells were seeded onto 96-well plates at a density of 10 000 cells per well in 100 μl of growth medium. At 50% confluence, cells were then treated during 24 h with the test compounds. After treating, cells were washed twice with 200 μl HBSS and were then incubated with 10 μM 2′,7′-dichlorofluorescein diacetate DCFH-DA (Sigma) in HBSS (Gibco) at 37 °C for 30 min. After loading, cells were washed twice with 200 μl HBSS in order to remove excess fluorescent dye, and then 100 μl HBSS/well were added. The fluorescence intensity of DCFH-DA was measured at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Relative changes in ROS concentration were determined by calculations of $\Delta F/F_0$, where $\Delta F/F_0 = (F_t - F_0)/F_0$. F_t represents the fluorescence reading at each time point and F_0 the initial fluorescence. Results were normalized to the protein content, estimated by using the method of Bradford [34].

2.5. Reporter plasmids transfections

Cell transfections were performed with the pNQO1-ARE-luciferase or with the mutated pNQO1-ARE-luciferase plasmid and Xtremegene HP transfection reagent from Roche Applied Science Diagnostics (Mannheim, Germany) according to the manufacturer's protocol. After 5 h of incubation, cells were treated during 24 h. Thereafter cells were lysed with Glo lysis buffer