Activation of AMP-kinase by AICAR induces apoptosis of DU-145 prostate cancer cells through generation of reactive oxygen species and activation of c-Jun N-terminal kinase

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Abstract. The growth of cancer cells is limited by energy supply which is regulated by the energy sensor AMP-kinase (AMPK). Hence, mimicking a low energy state may inhibit cancer growth and may be exploited in anticancer therapies. In the present study, the impact of AMPK activation on cell growth and apoptosis of DU-145 prostate cancer cells was investigated. Incubation with the AMPK activator aminoimidazole carboxamide ribonucleotide (AICAR) dose-dependently inhibited cell growth, activated AMPK, and inhibited mTOR. Furthermore, AICAR treatment activated c-Jun N-terminal kinase (JNK) and caspase-3, thereby initiating apoptosis. Within 60 min of treatment AICAR raised intracellular reactive oxygen species (ROS) which could be abolished in the presence of the free radical scavenger N-(2-mercaptopropionyl)glycin (NMPG), the AMPK inhibitor compound C (Comp C) and the respiratory chain complex I inhibitor rotenone, but not by the NADPH oxidase inhibitor VAS2870. Inhibition of ROS generation abolished AMPK activation by AICAR as well as JNK and caspase-3 activation. Furthermore, AMPK activation, JNK phosphorylation and cleaved caspase-3 upon AICAR treatment were abolished in the presence of Comp C. In summary, our data demonstrate that activation of AMPK by AICAR induces apoptosis of prostate cancer cells by a signaling pathway involving ROS, activation of JNK and cleaved caspase-3.

Introduction

In contrast to non-malignant cells, cancer cells display high rates of anabolic metabolism, over-express lipogenic enzymes, including acetyl-CoA carboxylase and fatty acid synthase, show enhanced protein biosynthesis, and are more active in synthesizing DNA. All these anabolic processes require energy and hence are expected to be regulated by the energy status of the cell, which is reflected in the cellular ATP/AMP ratio (1). Under conditions of metabolic stress, e.g., hypoxia, glucose deprivation or exercise which decrease intracellular ATP and increase AMP levels, the energy-sensing serine/threonine kinase AMPK is activated which then suppresses ATP-consuming metabolic functions and increases ATP-producing activities such as glucose uptake, fatty acid oxidation and mitochondrial biogenesis to restore energy homeostasis (2). AMP levels control AMPK enzyme activity by binding to the γ-subunit to cause conformational changes and allosteric activation thus rendering AMPK less prone to dephosphorylation by protein phosphatases (3). The activation of AMPK results in tumour suppression since the cell growth associated mTOR pathway is inhibited through direct phosphorylation of tuberous sclerosis complex 2 protein tuberin and the mTOR-associated factor Raptor, and cell cycle arrest or apoptosis is induced through phosphorylation of p53 and FOXO3a (4,5). Recently it was shown that inhibitors of glycolysis that are used to suppress tumour growth, activate AMPK, inhibit mTOR signaling and sensitize cells to death receptor (DR)-induced apoptosis (6).

The opportunity to fight cancer by interfering with the energy metabolism of cancer cells has resulted in several experimental studies that used AMPK activators like the guanidine derivative metformin or AICAR, which is taken up into cells by adenosine transporters. In the cytoplasm AICAR is converted by adenosine kinase to the non-phosphorylated derivative aminoimidazolecarboxamide ribonucleotide (ZMP), an analogue of 5'AMP. AICAR therefore mimics the cellular effects of AMP including AMPK activation. AICAR has been proven to possess anti-tumour properties in tumour cell cultures such as renal carcinoma cells (7), melanoma cells (8), retinoblastoma cells (9), glioblastoma cells (10), lymphoblastic leukemia (ALL) cells (11), and prostate cancer cells (12), however its mechanism of action is not sufficiently investigated. Recently it was demonstrated that AICAR increases oxidative stress in several cancer cell lines including HEPG2 hepatoma cells (13) and glioma (14). Furthermore, AICAR has been shown to activate JNK in

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pancreatic β cells (15). JNK is well known to be involved in oxidative stress-associated pro-apoptotic processes (16).

The present study was undertaken to unravel the mechanisms of the cytostatic effects of AMPK on prostate cancer cells. Our data demonstrate that ROS derived from the mitochondrial respiratory chain are the mediators of AICAR-induced apoptosis of DU-145 prostate cancer cells.

Materials and methods

Materials. Aminoimidazole carboxamide ribonucleotide (AICAR), compound C (Comp C), rotenone and JNK inhibitor II were purchased from Calbiochem (Bad Soden, Germany). VAS2870 was kindly provided by Vasopharm (Würzburg, Germany).

Cell culture. The human prostate cancer cell line DU-145 was used throughout the whole study. The cell line was grown routinely in 5% CO_2, humidified air at 37˚C with Ham's F-10 medium (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (Sigma, Deisenhofen, Germany), 2 mM glutamine, 0.1 mM β-mercaptoethanol, 2 mM minimal essential medium, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Karlsruhe, Germany).

Measurement of ROS generation. Intracellular ROS levels were measured using the fluorescent dye 2'7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Molecular Probes, Eugene, OR), which is a non-polar compound that is converted into a non-fluorescent polar derivative (H_2DCF) by cellular esterases after incorporation into cells. H_2DCF is membrane impermeable and is rapidly oxidized to the highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of intracellular ROS. For the experiments, DU-145 prostate cancer cells were incubated in E1 medium (containing in mM: NaCl 135, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, glucose 10, HEPES 10 (pH 7.4 at 23˚C), and 20 μM H_2DCF-DA dissolved in dimethyl sulfoxide (DMSO) was added. After 20 min, intracellular DCF fluorescence (corrected for background fluorescence) was evaluated in 3600 μm^2 regions of interest using an overlay mask unless otherwise indicated. For fluorescence excitation, the 488-nm band of the argon ion laser of a confocal laser scanning microscope (Leica SP2 AOBS, Bensheim, Germany) was used. Emission was recorded using a longpass LP515-nm filter set.

Immunohistochemistry. Cells grown on glass coverslips were washed with phosphate-buffered saline (PBS), fixed for 20 min at room temperature in 4% paraformaldehyde, washed again in PBS and fixed for 20 min in methanol (100%) at -20˚C. Subsequently cells were washed 3 times in PBS supplemented with 0.01% Triton-X-100 (Sigma) (PBST) and incubated for 45 min in 10% fetal calf serum dissolved 0.01% PBST to avoid unspecific binding. Thereafter cells were again washed 3 times in PBST 0.01% and incubated with primary antibody overnight at 4˚C. After washing 3 times in 0.01% PBST, secondary antibody staining was performed for 45 min. Primary antibodies used were: monoclonal rabbit anti phospho-AMPKα, monoclonal anti-phospho-mTOR, polyclonal anti phospho-SAPK/JNK, monoclonal anti cleaved caspase-3 (all from Cell Signaling, Frankfurt, Germany), used at a dilution of 1:100.

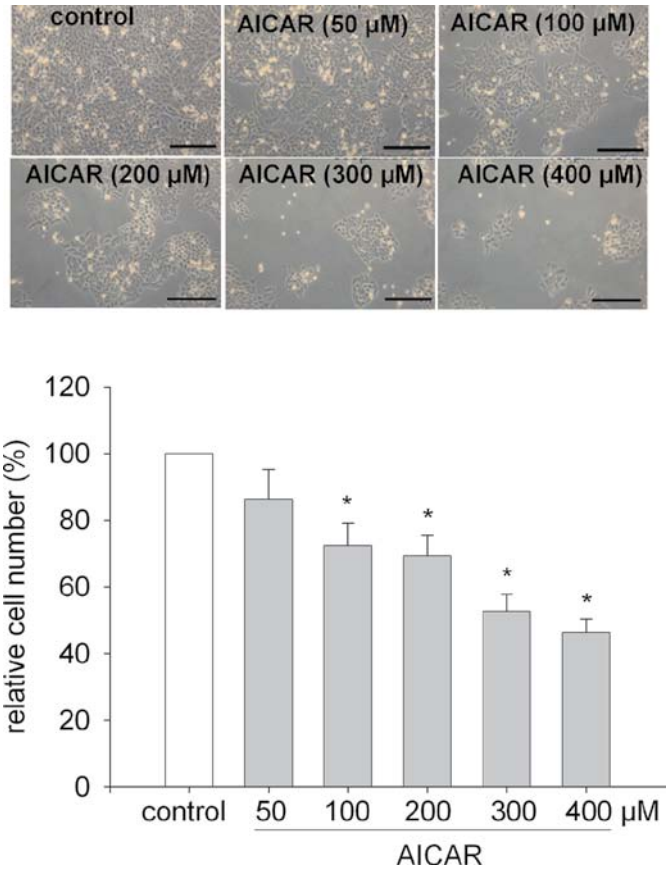

Figure 1. Dose response curve of the cytotoxic effects of AICAR. Monolayer cultures of DU-145 prostate cancer cells were treated for 4 days with increasing concentrations of AICAR. Subsequently cell numbers were analyzed by FACS. The upper panel shows representative transmission images of cell cultures treated by AICAR. The bar represents 120 μm. The lower panel shows cell numbers of AICAR treated cells upon AICAR treatment.

As secondary antibody a Cy5 goat anti-rabbit IgG (Dianova, Hamburg, Germany) was used in a dilution of 1:100.

Statistical analysis. Data are given as the mean values ± SD, with n denoting the number of experiments unless otherwise indicated. One-way ANOVA for unpaired data was applied as appropriate. $P<0.05$ was considered significant.

Results

AICAR inhibits proliferation of DU-145 prostate cancer cells. The aim of the present study was to investigate whether AICAR exerts anti-proliferative activity in prostate cancer cells via AMPK actiation. We therefore treated DU-145 prostate cancer cells grown in sub-confluent monolayer culture for 4 days in cell culture medium supplemented with increasing doses of AICAR (50-400 μM). Subsequently cells were enzymatically dissociated and cell numbers were analyzed by fluorescence activated cell sorting (FACS). Treatment with AICAR resulted in dose-dependent decline in cell numbers which reached ~50% of the cell number in the sample treated with 400 μM AICAR as compared to the untreated control (Fig. 1, n=3).

Activation of AMPK and mTOR upon treatment of DU-145 tumour cells with AICAR. AICAR is well known as an activator

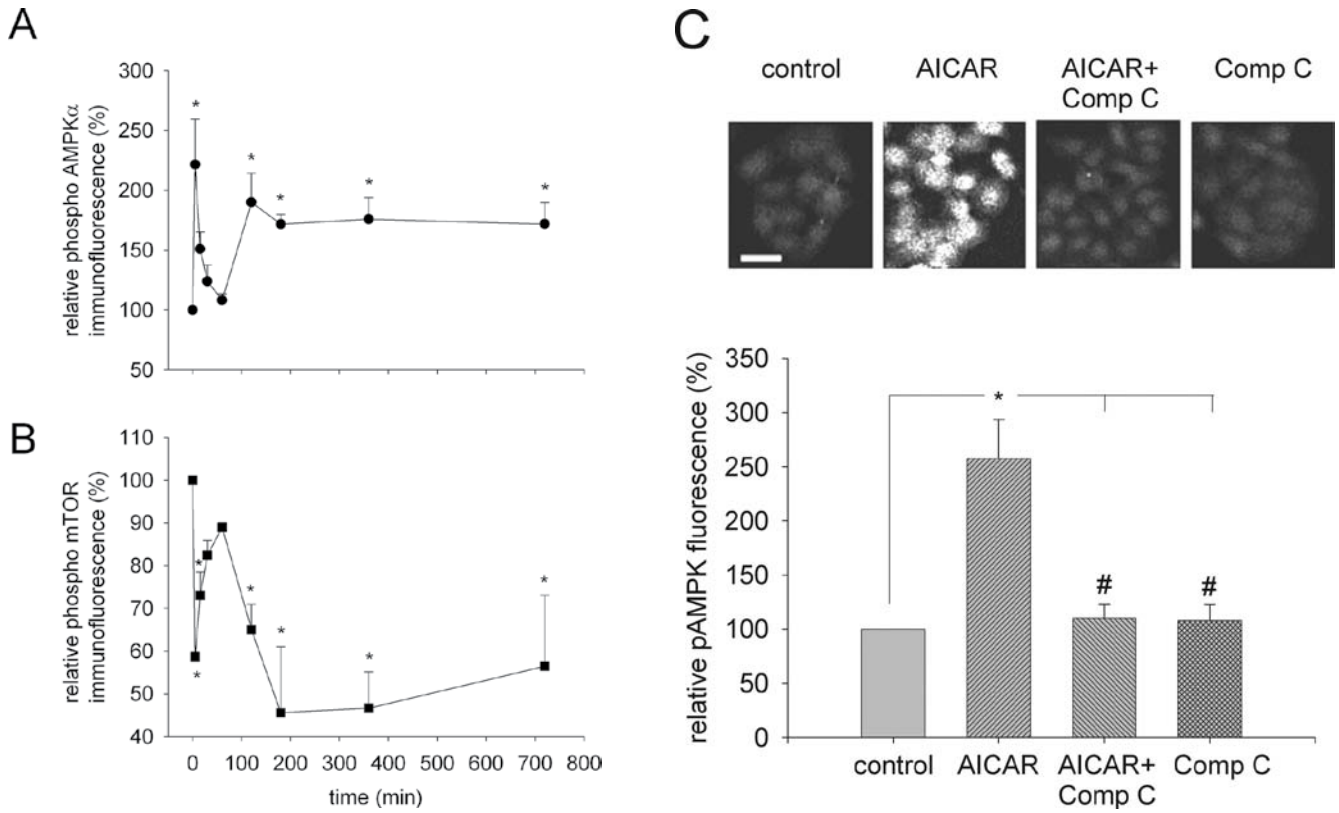

Figure 2. Time course of AMPK (A) and mTOR (B) activity upon treatment of DU-145 cells with AICAR (400 μM). Phosphorylation was monitored by the use of phosphor-specific antibodies and confocal laser scanning microscopy. Note that treatment with AICAR resulted in two phase AMPK activation which was paralleled with a two phase inactivation of mTOR. *P<0.05, significantly different as compared to the value at time 0. (C) The AMPK inhibitor Comp C (300 nM) completely abolished AICAR induced AMPK phosphorylation. The bar represents 35 μm. *,#P<0.05, significantly different as indicated.

of AMPK. On the other hand AMPK activation should result in downregulation of mTOR which is involved in the maintenance of cell growth. To investigate this point, the activation of the catalytic α-subunit of AMPK as well as mTOR was analyzed by using phospho-specific antibodies and confocal laser scanning microscopy. Treatment of sub-confluent DU-145 cancer cells resulted in significant AMPK activation within 5 min which was followed by downregulation towards the control value within the subsequent 60 min. After that AMPK was reactivated and remained on a significantly activated level for at least 700 min of AICAR treatment (Fig. 2A, n=3). Interestingly the activation of mTOR followed an approximately mirror-imaged time sequence with downregulation of mTOR activity within 5 min, reactivation within the subsequent 60 min and a further decrease in phosphorylation for the subsequent 700 min (Fig. 2B, n=3). To determine whether AMPK activation was indeed due to the addition of AICAR and not due to unspecific effects arising from medium change, AICAR was administered in either the presence or absence of the AMPK inhibitor Comp C. Under these conditions, activation of AMPK was totally blunted whereas basal phosphorylation of AMPK remained unaffected (Fig. 2C, n=3).

Activation of JNK by AICAR. AICAR has been previously shown to activate JNK in pancreatic β-cells (15), human neutrophils, and neuroblastoma cells (17). Using phospho-specific antibody against JNK it was demonstrated that JNK was transiently activated within a time frame of 120 min of AICAR (400 μM) treatment with maximum activation achieved after 60 min (Fig. 3A, n=3). Furthermore, a nuclear translocation of activated JNK into cell nuclei was observed by assessment of nucleus/cytoplasma fluorescence ratios (data not shown). When AICAR was coadminstrated with the AMPK inhibitor Comp C (300 nM) activation of JNK was totally abolished (Fig. 3B, n=3) which clearly demonstrates that JNK phosphorylation was dependent on AMPK activation by AICAR. In contrast to JNK only a moderate, non-significant activation of p38 MAPK activation was observed upon AICAR treatment (data not shown).

Activation of caspase-3 by AICAR. Treatment of several cancer cell lines with AICAR has been shown to cause apoptosis (18). However, it is not yet known by which signaling pathways apoptosis induction occurs. In the present study cleaved caspase-3, which is a key enzyme in the apoptotic signaling cascade, was assessed following 24 h of treatment with AICAR (400 μM) either in the presence or absence of Comp C (Fig. 4A and B, n=3). To investigate whether caspase-3 activation was downstream of JNK, AICAR was administered either in the presence or absence of JNK inhibitor II (10 μM) (Fig. 4A and C, n=3). Our data demonstrated that within 24 h of treatment cleaved caspase-3 was significantly upregulated which was totally abolished in either the presence of Comp C or JNK inhibitor II. This finding suggests that AICAR induces apoptosis via the AMPK and JNK pathway.

Generation of ROS following treatment of DU-145 cancer cells with AICAR. Cancer cells possess the capability of actively

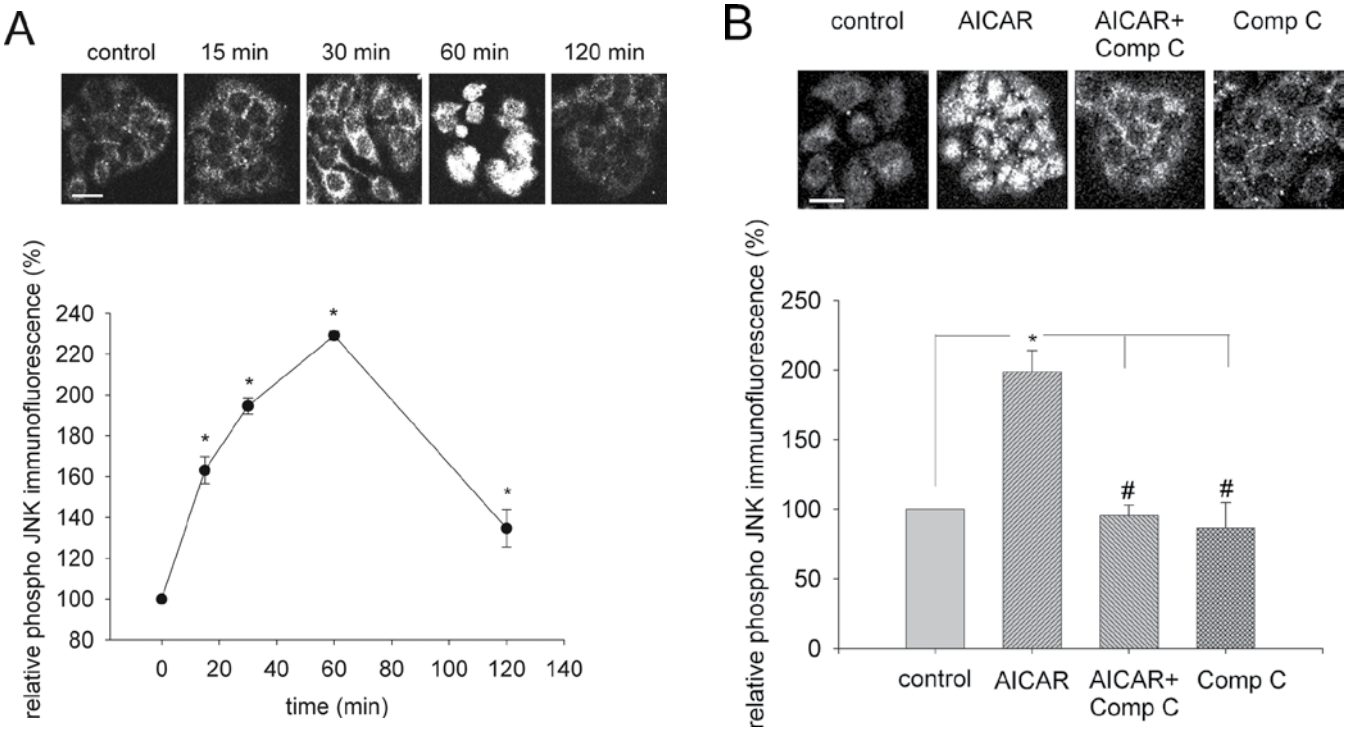

Figure 3. Activation of JNK by AICAR and effect of AMPK inhibition. (A) DU-145 prostate cancer cells grown in monolayer culture were incubated with AICAR (400 μM) which resulted in a transient activation of JNK as evaluated by confocal laser scanning microscopy using a JNK phosphor-specific antibody. The bar represents 35 μM. *P<0.05, significantly different as compared to the value at time 0. (B) Upon coadministration with the AMPK inhibitor Comp C (300 nM) JNK activation by AICAR was totally abolished. *,#P<0.05, significantly different as indicated.

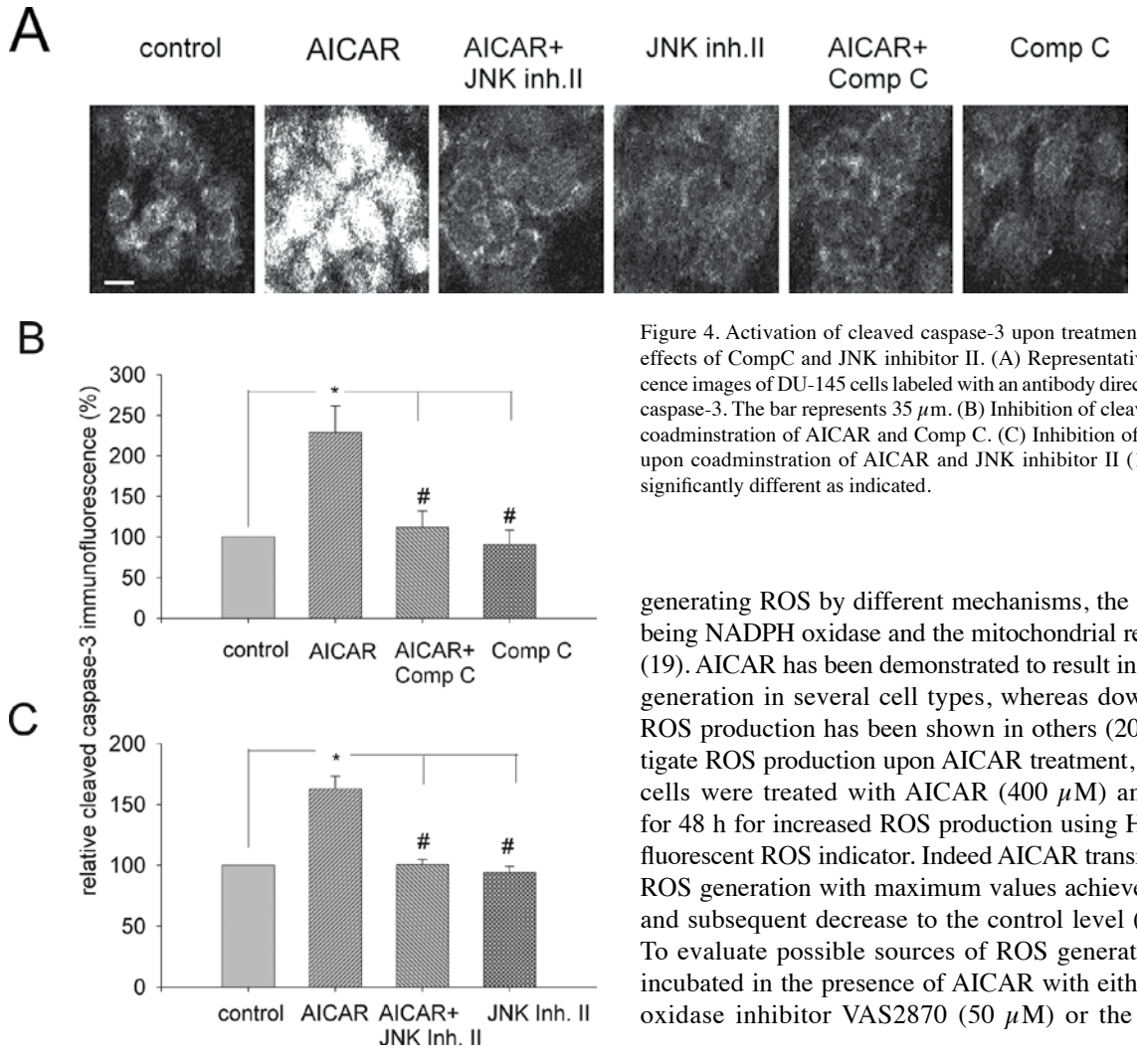

Figure 4. Activation of cleaved caspase-3 upon treatment with AICAR and effects of CompC and JNK inhibitor II. (A) Representative immunofluorescence images of DU-145 cells labeled with an antibody directed against cleaved caspase-3. The bar represents 35 μm. (B) Inhibition of cleaved caspase-3 upon coadminstration of AICAR and Comp C. (C) Inhibition of cleaved caspase-3 upon coadminstration of AICAR and JNK inhibitor II (10 μM). *,#P<0.05, significantly different as indicated.

generating ROS by different mechanisms, the most important being NADPH oxidase and the mitochondrial respiratory chain (19). AICAR has been demonstrated to result in increased ROS generation in several cell types, whereas downregulation of ROS production has been shown in others (20,21). To investigate ROS production upon AICAR treatment, prostate cancer cells were treated with AICAR (400 μM) and investigated for 48 h for increased ROS production using H_2DCF-DA as a fluorescent ROS indicator. Indeed AICAR transiently increased ROS generation with maximum values achieved after 60 min and subsequent decrease to the control level (Fig. 5A, n=3). To evaluate possible sources of ROS generation, cells were incubated in the presence of AICAR with either the NADPH oxidase inhibitor VAS2870 (50 μM) or the mitochondrial

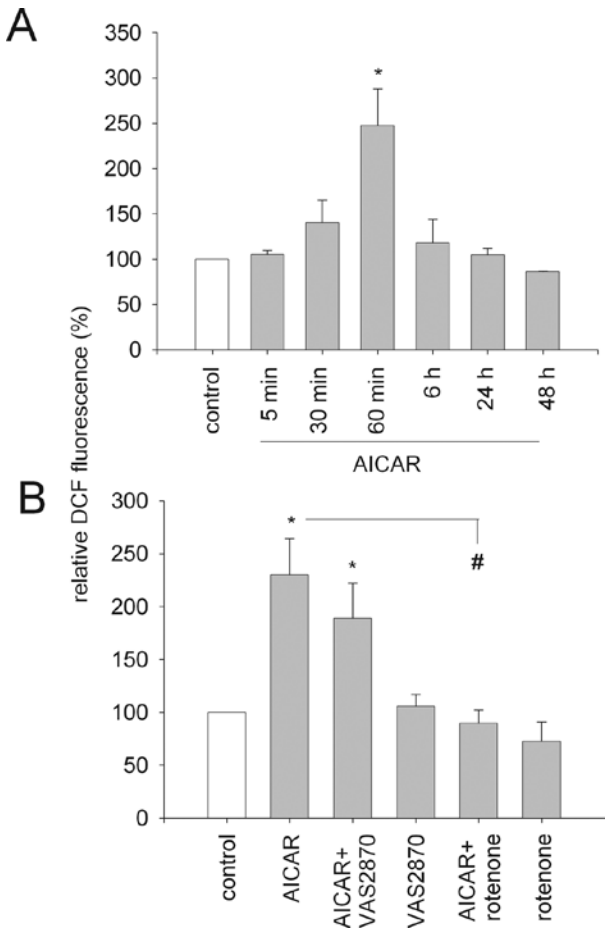

Figure 5. Generation of ROS upon treatment with AICAR (400 μM) and analysis of ROS sources. (A) Upon treatment with AICAR, ROS were transiently increased within 60 min. (B) Coadminstration of the NADPH oxidase inhibitor VAS2870 (50 μM) did not significantly decrease AICAR-induced ROS generation. In contrast the mitochondrial complex I inhibitor rotenone (2 μM) totally abolished the effect. $^{*,\#}$P<0.05, significantly different as indicated.

respiratory chain complex I inhibitor rotenone (2 μM) (Fig. 5B, n=3). The NADPH oxidase inhibitor VAS2870 had no effect on AICAR-induced ROS production, whereas under conditions of respiratory chain I inhibition the AICAR-induced elevation of ROS was totally abolished. This demonstrates that ROS generation upon AICAR treatment is mainly due to mitochondrial activity rather than activation of NADPH oxidase.

Control of AMPK activity, JNK and caspase-3 by ROS. The present study demonstrates that treatment of prostate cancer cells with AICAR increases ROS, and activates AMPK, JNK and caspase-3, leading to cell apoptosis. To investigate the meaning of ROS in the signaling cascade leading to apoptosis, we first investigated whether AMPK inhibition by Comp C would abolish AICAR-induced ROS generation (Fig. 6A, n=3). Secondly, we assessed whether the free radical scavenger NMPG (100 μM) would abolish AMPK activation (Fig. 6B, n=3). Thirdly, we determined whether inhibition of AMPK by Comp C would interfere with JNK activation Fig. 6C, n=3), and lastly, whether NMPG would inhibit cleaved caspase-3 expression (Fig. 6D, n=3).

Our experiments demonstrated that co-treatment of cells with AICAR and Comp C totally abolished ROS generation by AICAR, which indicates that AMPK activation is a prerequisite for ROS formation (Fig. 6A). These experiments were corroborated by the observation that in presence of the free radical scavenger NMPG, AMPKα phosphorylation was totally abolished (Fig. 6B). Likewise activation of JNK and expression of cleaved caspase-3 upon AICAR treatment were totally abolished in the presence of free radical scavenger, indicating that ROS are playing a primordial role in AICAR-mediated AMPK activity and JNK-mediated signaling pathways which finally lead to cancer cell apoptosis.

Discussion

Very recently the relation between cancer cell growth and cell metabolism has been pointed out, the energy regulating enzyme AMPK being one of its main players (22). Once activated, AMPK switches on catabolic pathways that generate ATP, while switching off ATP-consuming processes such as biosynthesis and cell growth and proliferation. This is achieved by down-regulation of mTOR signaling pathways which are critically involved in the maintenance of cell cycle activity. mTOR functions within the cell as a transducer of information from various sources, including growth factors, energy sensors, and hypoxia sensors, as well as components of the cell regulating growth and division. Blocking mTOR function mimics amino acid, and to some extent, growth factor deprivation, and exerts a cytostatic effect on proliferating cells *in vivo* (23). In current cancer trials, inhibition of mTOR is achieved by rapamycin and its derivatives. However, the clinical success of this therapy is limited by severe side effects of pharmacological mTOR inhibitors such as severe immunosuppression, thrombocytopenia and increase in blood fatty acids (23-25). In contrast indirect inhibition of mTOR through activation of AMPK can be achieved by relatively non-toxic agents like the blood glucose-lowering substance metformin or AICAR which through activation of AMPK improve blood glucose concentrations and lipid profiles, and thus are used in the treatment of type 2 diabetes and other metabolic disorders (26).

The present study extends the knowledge of the anti-proliferative activity of AICAR on cancer cells. Metformin was excluded from the present study due to the various side effects of this AMPK activator as mild inhibitor of mitochondrial respiratory chain complex 1 and its effects as an anti-oxidant (27). Our experiments confirm a recent study of Jose *et al* (18) who demonstrated the anti-proliferative activity of AICAR in several cancer cell lines, including DU-145 prostate cancer cells. In the present study we demonstrated that AICAR increased ROS production, activated JNK and increased cleaved caspase-3 levels thus initiating apoptosis pathways. In contrast to previous reports (28,29), only a marginal, non-significant activation of p38 MAPK was observed. Apparently apoptosis of DU-145 was due to AMPK activation and not to non-specific side effects of AICAR since caspase-3 activation as well as JNK phosphorylation was completely abrogated in presence of the AMPK inhibitor Comp C. The increase in ROS generation was not due to NADPH oxidase activation since the specific inhibitor VAS2870 was without effects. In the presents study the burst in ROS production occurred within 60 min of AICAR incubation and thus could not be due to increased mitochondrial proliferation which has previously shown to occur upon

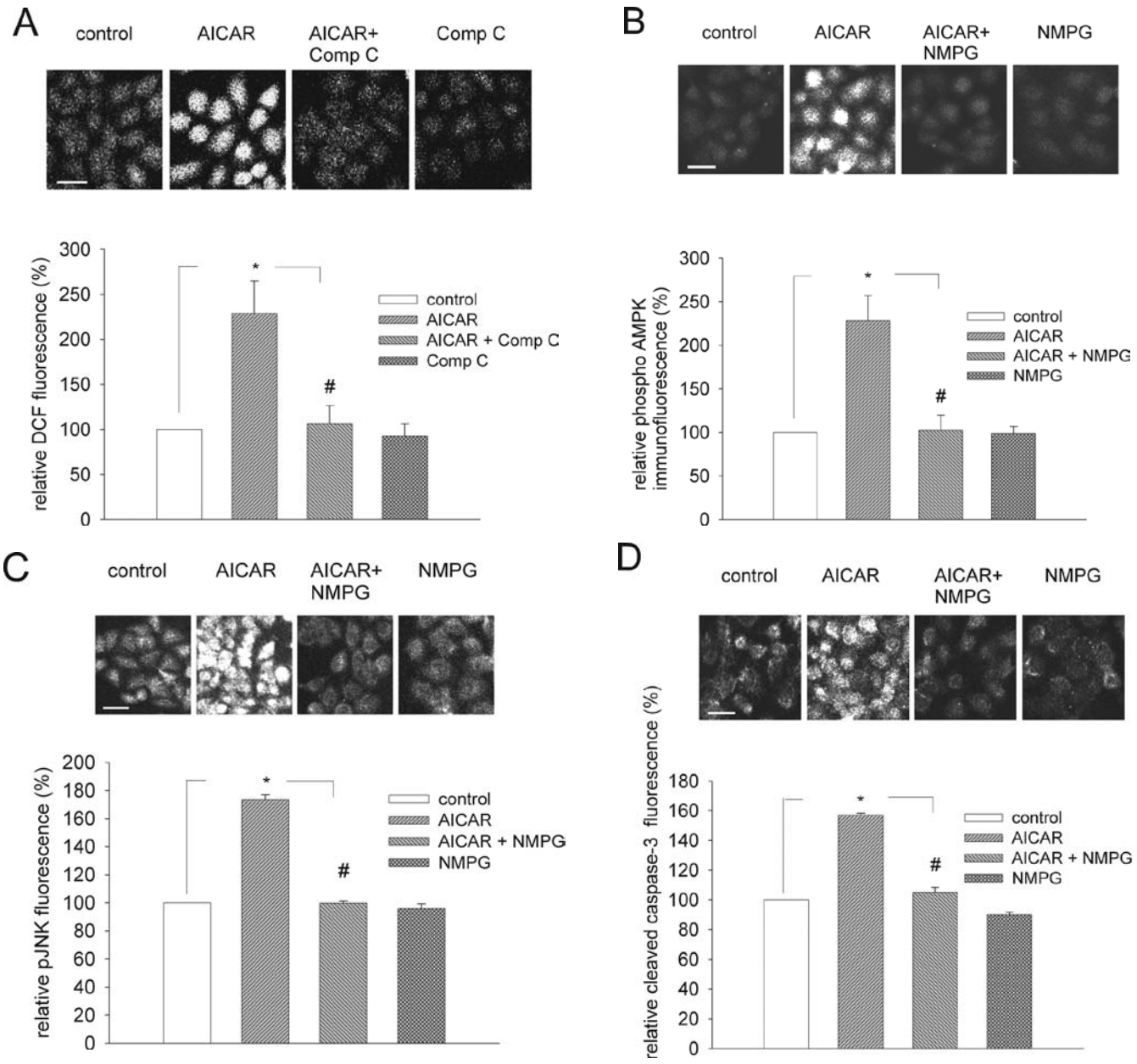

Figure 6. Effects of AMPK inhibition on ROS generation (A) and ROS scavenger on AMPK (B), JNK (C) and cleaved caspase 3 (D). (A) Inhibition of AMPK by Comp C totally abolished ROS generation by AICAR as evaluated by DCF fluorescence. (B-D) The free radical scavenger NMPG (100 μM) totally abolished AMPK (B) and JNK activation (C) as well as cleaved caspase-3 expression (D). The bar represents 35 μm. $^{*,\#}$P<0.05, significantly different as indicated.

long-term AICAR treatment (30). Presumably ROS production was due to activation of mitochondrial respiration since the complex I inhibitor rotenone completely abolished this effect. Increased mitochondrial respiration upon AICAR treatment has previously been demonstrated in skeletal muscle cells (31). Stimulated respiration should augment ROS generation which subsequently may activate the mitochondrial transition pore thus releasing cytochrome c and initiating apoptosis. However, the capacity of AICAR to stimulate ROS generation is still controversial. A decrease in ROS generation upon AICAR treatment has been observed in the aortic wall of ApoE$^{-/-}$ mice fed a high-fat diet (32), in skeletal muscle cells (33), HUVEC endothelial cells (34) and hepatic stellate cells (35). In contrast ROS elevation and induction of apoptosis were observed in several cancer cell lines including E47 hepatoma cells (13) and glioma (14) as well as in pancreatic β-cells (15). Thus, AICAR may trigger opposite responses in different cells, as previously discussed (18). To investigate the impact of ROS generation in AMPK, JNK and caspase-3 activation, cells were incubated either in the presence of JNK inhibitor II, the free radical scavenger NMPG or Comp C. In the presence of ROS scavenger, AMPK and JNK phosphorylation as well as caspase-3 activation were completely abolished which clearly indicates that ROS are regulating the signaling cascade leading to cell apoptosis. Upon JNK inhibition with JNK inhibitor II, AICAR-mediated caspase-3 expression was blunted thus demonstrating that JNK is indeed upstream of caspase-3. Furthermore, Comp C treatment abolished AICAR-induced JNK as well as caspase-3 activation, demonstrating that AICAR is acting in the sequence AMPK-JNK-caspase-3. AMPK activation through ROS has

been previously demonstrated among others in endothelial cells (36), HepG2 hepatocarcinoma cells (37) and vascular smooth muscle cells (38) which may occur before or without alteration in the ATP/AMP ratio (38-40). In this respect it has been recently shown that physiologically relevant concentrations of H_2O_2 can activate AMPK through oxidative modification of the AMPKα subunit, and it was discussed that AMPK activation, in addition to being a response to alterations in intracellular metabolic pathways, is directly influenced by cellular redox status (41). The data of the present study shed new light on the role of AMPK activity on cancer cell apoptosis. It may be speculated that under low, physiological, concentrations of AMPK activators a transient increase in AMPK activity occurs which regulates metabolic pathways, whereas high concentrations of AICAR and related compounds may initiate a vicious cycle of mimicking a persistent low energy state, prolongation of mTOR inhibition, mitochondrial dysfunction, oxidative stress and initiation of apoptotic pathways.

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