Published in final edited form as:

Cell Signal. 2011 September ; 23(9): 1466–1472. doi:10.1016/j.cellsig.2011.04.008.

AMPK-mediated autophagy is a survival mechanism in androgen-dependent prostate cancer cells subjected to androgen deprivation and hypoxia

Rishi Raj Chhipa, Yue Wu, and Clement Ip*

Department of Cancer Prevention and Control, Roswell Park Cancer Institute, Buffalo, NY 14263 USA

Abstract

The present study was designed to investigate (i) the role of AMPK activation in inducing autophagy in androgen-dependent prostate cancer cells subjected to androgen deprivation and hypoxia, and (ii) whether autophagy offers a survival advantage under these harsh conditions. Low androgen and low oxygen are two co-existing conditions frequently found in prostate cancer tissue following surgical or medical castration. In LNCaP cells, androgen deprivation and hypoxia together boosted AMPK activation to a higher level than that seen with either condition alone. The augmented AMPK response was associated with improved viability and the induction of autophagy. These observations suggest that a threshold of AMPK activity has to be attained in order to trigger autophagy, since neither androgen deprivation nor hypoxia by itself was capable of pushing AMPK activity past that threshold. Beclin-1 was identified as a potential downstream target of AMPK in turning on the autophagic cascade. If autophagy was blocked by chemical inhibition or RNA interference of key regulators, e.g., AMPK or beclin-1, more cells would die by apoptosis. The occurrence of autophagy is thus a survival mechanism for androgen-dependent prostate cancer cells to escape from an androgen-deprived and hypoxic subsistence.

Keywords

AMPK; hypoxia; autophagy; prostate cancer; androgen deprivation

1. Introduction

Tumor hypoxia is increasingly being recognized as an indicator of poor prognosis in prostate cancer [1]. The finding suggests that hypoxia may give rise to certain molecular changes which favor transformation to a more malignant phenotype. Androgen deprivation therapy, which is commonly used in treating advanced prostate cancer, also induces hypoxia in the tumor microenvironment, most likely due to a concomitant degeneration of the local vasculature [2]. As a general rule, reduced androgen availability and hypoxia are two closely associated conditions that exist simultaneously in the cancerous prostate tissue following

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*Correspondence and requests for reprints: Clement Ip, Ph.D., Department of Cancer Prevention and Control, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263 USA, Phone: 1-716-845-8875, Fax: 1-716-845-8100, clement.ip@roswellpark.org.

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surgical or medical castration. Not to be forgotten is the range of metabolic stress that emerges from the shadow of these debilitating forces, since androgen is critically involved in the transport of nutrients into prostate cells as well as the regulation of many anabolic processes inside the cells [3]. Thus in the absence of androgen, prostate cancer cells are faced with the challenge of dealing with a plethora of insults which threaten their viability.

Although androgen ablation results in prostate cancer regression initially, it loses effectiveness after a while. Relapse occurs and the cancer becomes androgen refractory or castration resistant. Apparently a subset of cancer cells is able to escape from an androgen-starved subsistence and continue to thrive. Our group reported previously [4] that AMP-activated protein kinase (AMPK) is an important signaling molecule which helps prostate cancer cells survive through the kind of metabolic stress generated by androgen removal. AMPK is an intracellular energy sensor whose main function is to block ATP-consuming processes and stimulate ATP-producing processes [5]. For example, in the above metabolic stress situation, the phosphorylation status of two immediate AMPK downstream effectors, acetyl CoA carboxylase (ACC) and mammalian target of rapamycin (mTOR), was modified in such a way as to depress their activities [6,7]. ACC is a key enzyme in fatty acid biosynthesis, while mTOR is involved in regulating protein synthesis and growth. By inhibiting the activity of these anabolic regulators, AMPK enables cells to conserve ATP for survival.

AMPK-dependent autophagy could be deemed as another energy conservation measure [8]. Autophagy recycles amino acids from self-digested organelles. A bare minimum of the most essential proteins may be produced so that cells can sustain themselves during a time of scarcity before new mechanisms set in to circumvent the dependence on androgen. The present study was designed to answer a number of related questions. First, do androgen deprivation and hypoxia together boost AMPK activity to a higher level compared to either treatment alone, and what would be the biological significance of the amplified response if it were to happen? Second, does an augmented increase of AMPK activity lead to autophagy, and if so, what might be a potential mediator linking AMPK to autophagy? Third, will the induction of autophagy offer a survival advantage to cells subjected to androgen deprivation and hypoxia? All the experiments were carried out in LNCaP human prostate cancer cells. LNCaP is a fast growing, androgen-dependent cell line. The model is therefore suitable to study how androgen deprivation and hypoxia modulate certain acute responses which impact on survivability.

2. Materials and methods

2.1. Cell culture

The LNCaP human prostate cancer cell line was obtained from the American Type Culture Collection, Manassas, VA. Cells were routinely cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mmol/L of glutamine, 100 units/mL of penicillin, and 100 µg/mL of streptomycin at 37°C in an atmosphere of 5% CO₂ and 95% air. The cell line was passaged for no more than three months or 10 passages. Before hypoxia treatment, cells were cultured in RPMI 1640 medium with 10% charcoal-stripped FBS (Hyclone) and supplemented with 1 nM testosterone (Sigma) for 48 h.

2.2. Hypoxia treatment

The culture medium was replaced with deoxygenated RPMI 1640 before hypoxia treatment, as reported previously [9]. The deoxygenated medium was prepared immediately prior to each experiment by equilibrating the medium with an hypoxic gas mixture containing 5% CO₂, 85% N₂, and 10% H₂ at 37°C. The oxygen concentration in the hypoxic chamber and

in the medium, which was monitored continuously by using an oxygen indicator (Forma Scientific), was maintained at <0.05%. All experiments were performed at 70% to 80% confluency at a medium pH between 7.2 and 7.4.

2.3. Chemical inhibitors

Compound C, obtained from Sigma, was used to inhibit AMPK activation at a concentration of 10 μ M. Dimethylsulfoxide was added as the vehicle for the untreated control. 3-Methyladenine (3-MA), purchased from Sigma, was used to inhibit autophagy at a concentration of 10 mM. Dimethylformamide was added as the vehicle for the untreated control.

2.4. AMPK α 1 knockdown by siRNA

All materials for siRNA transfection were purchased from Ambion (Austin, TX). Transient transfection of siRNA was done using a protocol recommended by the manufacturer. The AMPK α 1 siRNA sequences, which were accessed from the Ambion pre-designed siRNA library (ID number s100), were as follows: sense: 5'-GGAUCCAUCAUAUAGUUCAtt-3'; antisense: 5'-UGAACUAUAUGAUGGAUCctc-3'. Non-silencing siRNA sequence was used as negative control. All siRNAs, obtained in lyophilized and annealed form, were resuspended in diethylpyrocarbonate-treated distilled water to achieve a stock concentration of 20 μ M, and stored at -20°C in 50- μ l aliquots. LNCaP cells were plated onto 6-well plates to achieve 60% confluency. Lipofectamine 2000 transfection reagent (Invitrogen) and fresh medium containing 40 nM of either negative control or AMPK α 1 siRNA were incubated in separate tubes for 5 min. The transfection reagent and siRNA solution were then combined and incubated for 30 min at room temperature to allow complex formation. The siRNA-transfection reagent complexes were added drop-wise to each well of cells bathed in 2 ml of fresh medium. In some experiments, LNCaP cells, which stably expressed GFP-LC3, were used. The pEGFP-LC3 plasmid was obtained as a gift from Tamotsu Yoshimori, Osaka University, Japan [10]. Transfected cells were cultured in an hypoxic chamber and harvested for survival, imaging and Western blot analyses.

2.5. Cell survival and cell death analyses

Cell survival analysis was performed by using the MTT assay, as described in our previous publication [4]. The data are expressed as optical density (OD) units. Cell death analysis was performed by using the Cell Death Detection ELISA kit from Roche Applied Science (Indianapolis, IN). This method quantifies apoptotic death by determining the presence of cytoplasmic histone-associated DNA fragments. Cell death analysis was carried out in 96-well plates. For each treatment, 3 wells of cells were used. The DNA fragmentation reading (measured in OD unit) was then normalized against the protein concentration in either normoxic or hypoxic condition.

2.6. Western blot analysis

Equal amounts of protein were analyzed in duplicate by SDS-PAGE. Protein concentrations were measured by the BCA protein assay kit as per manufacturer's protocol (Pierce). Antibodies to AMPK α , phospho-AMPK α (Thr172), HIF1- α , p62, LC3, Beclin-1, ATG5, ATG12, and PARP were purchased from Cell Signaling Technology (Beverly, MA). Anti- α -tubulin was obtained from Sigma and anti-GAPDH was from Santa Cruz Biotechnology. Immunoreactive proteins were detected with a HRP-conjugated secondary antibody (Biorad) and visualized by using an enhanced chemiluminescence detection system (Amersham Bioscience).

2.7. Beclin-1 knockdown by shRNA

Two BECN1 shRNA constructs were tested from the GIPZ lentiviral shRNAmir library developed by Open Biosystems. These were hairpin sequence for V2LHS_23692 (denoted as BECN1 sh1):

TGCTGTTGACAGTGAGCGACCGTGGAATGGAATGAGATTATAGTGAAGCCACA
GATGTATAATCTCATTCCATTCCACGGGTGCCTACTGCCTCGGA, and hairpin
sequence for V2LHS_241693 (denoted as BECN1 sh2):

TGCTGTTGACAGTGAGCGAGCCAATAAGATGGGTCTGAAATAGTGAAGCCACA
GATGTATTTTCAGACCCATCTTATTGGCC TGCCTACTGCCTCGGA. Lipofectamine

2000 transfection of shRNA-pGIPZ plasmid, psPAX2 or pCMV-dR8.74 (plasmid containing *gag*, *pol* and *rev* genes), pMD2.G (VSV-G expressing envelope plasmid) and pGIPZ non-silencing scrambled control plasmid was used in HEK293T cells to generate lentiviral transduction particles. After 48 h, the virus-containing supernatant was harvested by filtration through 0.45 μ m cellulose acetate (low protein binding) syringe filter. LNCaP cells were infected with viral supernatant by using 4 μ g/ml of polybrene. The resistant clones were selected by replacing the puromycin selection media (2 μ g/ml) every 3–4 days. Cells were then tested for beclin-1 knockdown. Our results showed that BECN1 sh2 worked much better than BECN1 sh1 in decreasing beclin-1 expression.

2.8. Statistical analysis

The Student's t-test was used to determine statistical difference between treatment and control values. A P value of <0.05 is considered significant.

3. Results

3.1. Augmented AMPK activation following androgen deprivation and hypoxia – an association with improved viability

LNCaP cells were subjected to androgen deprivation in the presence of normoxia or hypoxia. Control androgen-supplemented cells were treated with 1 nM testosterone. AMPK activation was monitored by its phosphorylation status at 24, 48 or 72 h. The Western blot results are shown in Fig. 1A. Androgen deprivation alone caused a small increase of phospho-AMPK α in normoxic cells. Hypoxia alone (without androgen deprivation) produced a slightly larger increase of phospho-AMPK α . In contrast, androgen deprivation and hypoxia together gave rise to a much more robust response at both 24 and 48 h. Total AMPK α remained unchanged in all the experimental conditions. Hypoxia was confirmed by the strong expression of HIF1 α in the cells.

Viability of LNCaP cells subjected to androgen deprivation, with or without hypoxia, was assessed by the MTT assay (Fig. 1B). Cells cultured in 1 nM testosterone and normoxia nearly doubled in number over a 4-day period. In the absence of androgen, growth was reduced significantly. These two conditions were included to confirm the androgen-dependent nature of the model. In hypoxia, cells cultured without testosterone actually fared better than those with testosterone. There were more viable cells in the former group at day 4 ($P < 0.05$). The above experiments suggest that an amplified AMPK activation signal might serve as an indemnity against the damage inflicted by androgen deprivation and hypoxia together.

3.2. Survival benefit of AMPK activation in androgen deprivation and hypoxia

In order to address the role of AMPK in survivability, an experiment was carried out in which Compound C (chemical name is 6-[4-(2-Piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine) was used to block AMPK activation at a concentration of 10 μ M, as described in our previous publication [4]. Compound C is a potent, selective ATP-

competitive inhibitor of AMPK. The attenuation of phospho-AMPK α was confirmed in cells cultured in hypoxia, with or without testosterone (Fig. 2A). The effect of Compound C on cell viability is shown in Fig. 2B. Compound C did not seem to affect cell viability in an hypoxic condition when testosterone was present. However, in the absence of testosterone, Compound C was found to have an adverse effect on cell viability. The finding suggests that AMPK activation is critical if cells were to survive the punishment of androgen deprivation and hypoxia.

3.3. Induction of cytoprotective autophagy by androgen deprivation and hypoxia

Autophagy was assessed by several well accepted molecular markers, as determined by Western blot analyses. One of the specific substrates degraded during autophagy is p62 [11,12]. Figure 3A compares the level of p62 in normoxia and hypoxia, with or without testosterone. A slight decrease of p62 was detected in hypoxia alone. However, when hypoxia was accompanied by testosterone withdrawal, a more pronounced decrease was observed after 48 or 72 h. The data suggest the occurrence of autophagic protein degradation under the above condition. Similarly, the level of LC3-II was much more pronounced in cells deprived of androgen and oxygen than in those deprived of oxygen alone (Fig. 3B). LC3-I and LC3-II represent the same protein, with the exception that LC3-II also contains phosphatidylethanolamine. LC3-II is the component that is recruited to the autophagosome membrane. The ratio of LC3-II to LC3-I is shown in a bar graph format directly below each of the LC3 Western blot lanes. The increase of LC3-II/LC3-I ratio in hypoxia and testosterone removal was clearly evident in day 1 and day 2. The drop of the day 3 ratio may indicate an accelerated LC3-II turnover or degradation due to a prolonged autophagy [13]. The Atg5-Atg12 complex, which is also found in the autophagosome membrane [14], was similarly increased to a higher level in an androgen- and oxygen-starved condition than in hypoxia alone. Beclin-1 is a positive regulator of autophagy [15]. The level of beclin-1 remained stable with time in androgen-depleted and hypoxic cells. In contrast, beclin-1 fell to a much lower level in hypoxic cells supplemented with androgen. The blots shown in Fig. 3B are representative. The experiment was repeated 3 times. The same trend was observed. The conclusion from the autophagy marker data is that there appeared to be an increase of autophagic activity by androgen deprivation and hypoxia when compared to the single treatment.

In order to determine whether the induction of autophagy was cytoprotective or cytolytic, the same cell viability assay was performed in the presence of 3-methyladenine (3-MA), the latter is a known inhibitor of autophagy [13]. The inhibitor was found to reduce viability significantly in the androgen-deprived and hypoxic cell population (Fig. 3C). It had minimal effect in hypoxic cells supplemented with androgen. The ability of 3-MA to attenuate the expression of autophagic markers in androgen-deprived and hypoxic cells was confirmed by the results shown in Fig. 3D. The above experiments (repeated 3 times) suggest that autophagy is important to improving survivability of androgen-dependent prostate cancer cells subjected to androgen-deprivation and hypoxia.

3.4. Role of AMPK in autophagy induction

In order to delineate the role of AMPK activation in steering cells towards autophagy in an androgen- and oxygen-starved condition, an experiment was carried out in which Compound C was used to inhibit AMPK activation in normoxia or hypoxia, both in the absence of testosterone. Autophagosome formation was confirmed by the accumulation of punctate GFP-LC3 structures only in an oxygen- and androgen-deprived condition, as shown by the micrograph of Fig. 4A. In the presence of Compound C, no autophagosome was formed under the same condition. The results suggest two important points. First, testosterone removal alone (i.e. without hypoxia) is not sufficient to induce autophagy. Second, by

blocking AMPK activation with Compound C, autophagy cannot take place in the oxygen- and testosterone-deprived cells.

To further verify the role of AMPK in autophagy induction, another experiment was carried out in which siRNA was used to knock down the expression of AMPK α . The Western blot results of Fig. 4B show that the above method reduced the protein level of both total and phospho-AMPK α . The autophagic marker, LC3-II, was decreased to near non-detectable level by AMPK siRNA, suggesting that AMPK is upstream of the autophagic cascade. Induction of autophagy was also confirmed by the accumulation of punctate GFP-LC3 structures, as depicted in the micrograph of Fig. 4C. These structures (indicated by an arrow in the upper right panel), appeared only in the androgen-deprived and hypoxic cells. Knocking down AMPK α in these cells (lower right panel) did not allow the punctate structures to be formed. In the presence of androgen sufficiency, hypoxia alone also failed to induce significant autophagy. The percentage of autophagic cells under the various conditions, as determined by the fractional population with LC3 puncta, was quantified (Fig. 4D). About 4% of cells exhibited autophagy in an hypoxic, androgen-sufficient condition. This value rose to ~18% in androgen- and oxygen-deprived cells, the increase from 4% to 18% is statistically significant ($P < 0.05$). The proportion of autophagic cells fell back to ~8% with the knockdown of AMPK ($P < 0.05$).

3.5. Evidence of autophagy as a survival mechanism

The above experiments established the concept that AMPK is important for the induction of autophagy in androgen- and oxygen-deprived cells. If autophagy was not allowed to occur by knocking down AMPK, what would become the fate of these cells? Would they succumb to apoptosis? To address this question, changes in apoptotic death were evaluated by the DNA fragmentation assay in cells transfected with AMPK siRNA (Fig. 5A). Compared to normoxia, hypoxia caused a marked enhancement of DNA fragmentation regardless of the presence or absence of androgen. AMPK knockdown did not increase apoptosis in hypoxic cells supplemented with androgen. However, in androgen- and oxygen-deprived cells, AMPK knockdown significantly increased apoptosis. The finding suggests that if autophagy was blocked, more cells would die by apoptosis.

What could be a possible link between AMPK and autophagy? The next series of experiments was targeted on beclin-1 because it is a key regulator of the autophagic cascade. Another reason for focusing on beclin-1 was because beclin-1 stabilization was associated with androgen deprivation and hypoxia (see Fig. 3A). Knocking down AMPK in androgen-deprived and hypoxic cells was found to decrease the protein level of beclin-1, as shown by the Western blot data of Fig. 5B. To follow up on the AMPK-beclin connection and to prove that apoptosis is inevitable if there is no autophagy, another experiment was carried out in which beclin-1 knockdown was achieved by shRNA (Fig. 5C). As expected, LC3-II was reduced markedly by BECN1 shRNA in androgen-deprived and hypoxic cells. The enhanced LC3-II to LC3-I ratio was also nullified by BECN1 shRNA. More importantly, the loss of LC3-II was accompanied by an increase of PARP cleavage. The latter is a commonly used indicator of apoptosis. The experiment of Fig. 5C was performed 3 times. The same trend was observed in the repeat experiments.

4. Discussion

A major finding of the present study is that androgen deprivation and hypoxia together boost AMPK activation in androgen-dependent prostate cancer cells to a higher level than that seen with either condition alone. Furthermore, the augmented AMPK response is necessary to drive cells to autophagy in prolonging survival. These observations imply that there is a threshold of AMPK activity that has to be attained in order to trigger a significant induction

of autophagy. Neither androgen deprivation nor hypoxia by itself is capable of pushing AMPK activity past that threshold to switch on autophagy. A modest AMPK signal may call for the shutting down of ATP consuming processes, such as fatty acid and protein synthesis, as described in our previous publication [4]. It has been reported recently that phosphorylation of ULK1(hATG1) by AMPK connects energy sensing to mitophagy [12,16]. AMPK suppresses mTOR activity, and mTOR inhibits ULK1. AMPK may thus control ULK1 via a two-pronged mechanism, thereby ensuring an efficient coupling of energy stress, autophagy and cell survival. We have additional information that the activity of mTOR (associated with protein synthesis) was depressed by either androgen deprivation or hypoxia. We decided not to include the data here because we preferred to focus attention on the relationship between AMPK and autophagy in this paper. In contrast to a milder AMPK signal, a strong AMPK signal may represent an alarm to marshal the autophagy machinery as a last ditch effort to sustain cell viability.

Hypoxia has the potential to alter the growth, differentiation characteristics, and apoptotic sensitivity of prostate cancer cells [1]. It has been known for some time that hypoxia is a potent stimulus of AMPK and that AMPK activation may occur even before there are detectable decreases of intracellular ATP level [17,18]. Since androgen is an anabolic hormone for the proliferation of prostate cancer cells, the lack of androgen may cause AMPK activation via a different mechanism than that initiated by hypoxia. As noted in the Introduction, low androgen and low oxygen are two co-existing conditions frequently found in prostate cancer tissue following surgical or medical castration. These conditions may select for an androgen-independent phenotype with an improved survival advantage and a propensity to invade and metastasize [19]. The above argument is in line with our previous study demonstrating that prostate cancer cells which are adept in achieving a vibrant AMPK status are better equipped to propagate to androgen independence [4].

The positive regulation of autophagy by AMPK has been reported recently [20]. The detailed mechanism has yet to be worked out, although beclin-1 is beginning to emerge as a key mediator. In one study, it was found that cisplatin induction of beclin-1 and autophagy in glioma cells was impaired by AMPK siRNA transfection [21]. Another study reported that chemical activation of AMPK resulted in an up-regulation of beclin-1 expression in rat hepatoma cells, and that the process might involve the MEK/ERK signaling pathway [22]. The present study also identified beclin-1 as an important downstream target of AMPK in turning on the autophagic cascade in prostate cancer cells. Further investigation is currently underway to elucidate the molecular events leading to the stabilization of beclin-1 by AMPK.

Induction of autophagy by anticancer drugs has been widely studied in a number of cancer cell models [23–25]. Initially, autophagy was regarded as a form of cell death, since it is essentially a catabolic, self-cannibalizing event. However, an increasing body of evidence is pointing to the idea that autophagy may help cancer cells survive through chemotherapy and various kinds of physiological stress [15,26,27]. LNCaP cells have been known to resort to autophagy during androgen or serum starvation in enabling the transition from androgen dependence to androgen independence [15]. Our work added a new dimension to the above study in establishing the role of AMPK in inducing autophagy and the function of autophagy in rescuing cells from apoptosis. Our experiments clearly showed that if autophagy was blocked by RNA interference of key regulators, e.g. AMPK or beclin-1, cells would die by apoptosis. Contrary to the expectation that castration would leave behind more apoptotic cells in the residual tumor, there was actually a decrease of apoptotic index [28]. Although further studies are necessary to elucidate the underlying reason for the above observation, it is tempting to surmise that autophagy may be involved in saving some cells from apoptotic death. A successful therapeutic outcome is aimed not only at killing as many cancer cells as

possible, but also at barricading any possible escape route. In the case of prostate cancer, androgen deprivation therapy may need to be combined with a strategy to block autophagy in order to achieve a better remission response.

5. Conclusion

A high level of AMPK activation is necessary to trigger autophagy in androgen-dependent prostate cancer cells subjected to androgen deprivation and hypoxia. The induction of autophagy in this situation is meant to provide a survival advantage to the beleaguered cancer cells. The conclusion is supported by the evidence that if autophagy is blocked by RNA interference of key regulators, more cells would die from apoptosis.

Abbreviations

3-MA	3-methyl adenine
ACC	acetyl coenzyme A carboxylase
AMPK	AMP-activated protein kinase
Comp C	Compound C
GADPH	glyceraldehyde 3-phosphate dehydrogenase
mTOR	mammalian target of rapamycin
PARP	poly ADP-ribose polymerase
shRNA	short hairpin RNA
siRNA	small interference RNA

Acknowledgments

This work was supported by a grant from NIH/NCI P01 CA126804 (C. Ip, P.I.) and partially supported by shared resources of NIH/NCI P30 CA16056 (Roswell Park Cancer Center Support Grant).

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Fig. 1.

Effect of androgen deprivation and hypoxia on AMPK activation and cell viability. (A) Western blot of phosphorylated and total AMPK α in normoxia/hypoxia, and with or without 1 nM testosterone. The numbers indicate the relative protein level as determined by densitometry and normalized to total AMPK α . Hypoxia was confirmed by the expression of HIF1 α . (B) Cell viability as determined by the MTT assay. NX, normoxia; HX, hypoxia; +T, with 1 nM testosterone; -T, without 1 nM testosterone. *P<0.05 compared to the HX+T value.

Fig. 2. Effect of Compound C on AMPK activation and cell viability. (A) Compound C mediated inhibition of AMPK phosphorylation in hypoxic cells, with or without testosterone. (B) Compound C mediated inhibition of cell viability in androgen-deprived and hypoxic cells. * $P < 0.05$.

Fig. 3.

Induction of cytoprotective autophagy by androgen deprivation and hypoxia. (A) Western blot of p62 (a specific substrate of autophagic degradation) in normoxic or hypoxic cells, and with or without testosterone. (B) Western blot of LC3-II, Atg5-Atg12 and beclin-1 (all autophagic markers) in hypoxic cells, with or without testosterone. The ratio of LC3-II to LC3-I is shown in a bar graph format directly under each of the Western blot lanes. The numbers below the Atg5-Atg12 and beclin-1 Western blots indicate the relative protein level as determined by densitometry and normalized to GAPDH. (C) Effect of 3-methyladenine (3-MA) on cell viability in hypoxic cells, with or without testosterone. 3-MA is an inhibitor of autophagy. * $P < 0.05$ compared to the corresponding value in the presence of 3-MA. (D) 3-MA mediated inhibition of autophagic marker expression in androgen-deprived and hypoxic cells.

Fig. 4.

Role of AMPK in autophagy induction in androgen-deprived and hypoxic cells. (A) Effect of Compound C on GFP-LC3 puncta formation (indicated by arrow) in normoxic or hypoxic cells, both in the absence of testosterone. For interpretation of the color in the figure, the reader is referred to the web version of the article. (B) Effect of AMPK siRNA transfection on phospho-AMPK α and LC3 –II expression. (C) Inhibition of LC3 puncta formation in androgen-deprived and hypoxic cells by AMPK siRNA transfection. GFP-LC3 puncta (indicated by arrow) can be visualized in control siRNA transfected cells. For each micrograph shown, between 5 to 8 fields were observed in each experiment. (D) Percentage of autophagic cells, as calculated based on LC-3 puncta positive cells, in various conditions. $P < 0.05$, $n = 3$ independent experiments.

Fig. 5.

Role of autophagy as a survival mechanism. (A) Increase of apoptotic cell death, as measured by DNA fragmentation, in androgen-deprived and hypoxic cells by AMPK siRNA transfection. The experiment was performed in triplicate. * $P < 0.05$ compared to the corresponding value in the presence of testosterone. (B) Effect of AMPK siRNA transfection on beclin-1 expression in androgen-deprived and hypoxic cells. (C) Effect of BECN1 shRNA transfection on LC3-II expression and PARP cleavage in hypoxia, with or without testosterone. The ratio of LC3-II to LC3-I is shown in a bar graph format directly under each of the Western blot lanes. The numbers below the PARP Western blot indicate the relative level of cleaved PARP as determined by densitometry and normalized to GADPH.