Androgen Receptor-mTOR Crosstalk is Regulated by Testosterone Availability: Implication for Prostate Cancer Cell Survival

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Abstract. *Background: Signaling between androgen receptor (AR) and mTOR may be crucial for prostate cancer cells to endure the low androgen and suboptimal nutrient conditions produced by androgen deprivation therapy. Materials and Methods: AR and mTOR cross-talk was examined in LNCaP cells exposed to either high or low testosterone. AR and mTOR activities were modified separately using either siRNA knockdown or specific chemical inhibitor. The biological significance of the reciprocal communication was assessed by susceptibility to glucose deprivation-induced cell death. Results: AR positively regulated mTOR activity in both low and high testosterone levels. TSC1 and TSC2, the two negative regulators of mTOR, may be involved since both were up-regulated by AR knockdown. Sub-baseline mTOR increased AR protein levels. However, this effect only occurred with low testosterone. More cells underwent apoptosis if AR function was inhibited during glucose deprivation, which significantly depressed mTOR activity. Conclusion: The compensatory increase of AR function due to a repressed mTOR signal is advantageous for survival. Disrupting this loop at the time of initiation of androgen deprivation therapy may delay, or even prevent, the recurrence of prostate cancer.*

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Androgen deprivation therapy (ADT) is a treatment modality of choice for advanced prostate cancer or prostate cancer that recurs when prostatectomy or radiation fails. Most prostate tumors, however, become refractory to ADT after a period of remission and soon cause death. Delineating the mechanism responsible for the transition to "ADT resistance" is critical to the development of new strategies to block the emergence of this lethal phenotype. One physiological consequence of ADT is shrinking vasculature in the prostate cancer tissue (1). Against this backdrop, prostate cancer cells must maintain androgen receptor (AR) function in a low androgen environment, and endure the stress of a suboptimal supply of oxygen and nutrients. More knowledge is needed in order to understand why AR activity is essential for the management of post-ADT stress, how a vital nutrient-sensitive signaling pathway responds to testosterone changes, and whether there is cooperation between these two molecular machineries to help cells withstand the trauma of ADT.

The protein kinase mammalian target of rapamycin (mTOR) is a crucial signal transducer for cell growth and survival (2). Since mTOR activity is sensitive to the availability of glucose, nutrients, oxygen and growth factors, it serves as a key interface molecule between the cell and the microenvironment. mTOR does not act alone but binds to various subunits to form mTORC1 or mTORC2 complexes, which are known to carry out different functions. mTORC1 supports global protein translation by phosphorylating downstream effectors, such as p70S6 kinase, S6 ribosomal protein, and 4EBP-1 (2, 3). TSC1 and TSC2 (tuberous sclerosis complex 1 and -2) are negative regulators of mTORC1. Glucose or nutrient insufficiency dampens mTOR activity through TSC1 and TSC2 to reduce protein synthesis

(2). Low mTOR levels may also trigger the initiation of autophagy, so that cells are able to recycle nutrients by breaking down spare organelles (4).

The cross-talk between AR and mTOR may impact the transition of prostate cancer from androgen dependence to ADT resistance. A recent report suggested that androgen up-regulates mTOR activity *via* AR-mediated transcription of nutrient transporters (5). Additionally, two studies demonstrated that inhibition of mTOR by rapamycin produces an increase in the protein level or activity of AR (6,7). The above findings were observed in a culture condition with very low androgen levels. Little is known regarding how the cross-talk between AR and mTOR may behave in response to changes in the availability of exogenous androgen. The answer to this important question might provide valuable clues to the understanding of ADT resistance. Since testosterone is the major circulating androgen, the present study was designed to investigate the role of testosterone on the reciprocal communication between AR and mTOR. Additionally, the study also investigated the importance of AR activity in protecting cells from the stress of glucose deprivation and the accompanying down-regulation of mTOR.

Materials and Methods

Cell cultures. The LNCaP human prostate cancer cell line was obtained from the American Type Culture Collection, Manassas, VA, USA. The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin/streptomycin, and 2 mM glutamine. The cells were maintained at 37˚C in an atmosphere of 5% CO_2 and 95% air.

Low and high testosterone conditions. Nearly all androgen-responsive prostate cancer cell lines used in research, including the LNCaP cells in the host laboratory, are propagated routinely in a medium supplemented with 10% FBS. Commercial FBS contains approximately 0.3 nM testosterone (data provided by vendors, and also confirmed by HPLC-MS analysis). These androgen-responsive cells are thus accustomed to an environment of 0.03 nM testosterone. This level of testosterone is lower than what has been reported for circulating testosterone in castrated males (8). This traditional protocol is referred to as a 'low testosterone condition'. For a 'high testosterone condition', exogenous testosterone (Sigma, St. Louis, MO, USA) was added to the medium at a final concentration of 5 nM. Cells treated with testosterone in this way are used historically to study the acute effect of testosterone.

Drug treatment and glucose deprivation. Depending on the experimental design, bicalutamide (Sigma) or rapamycin (Calbiochem, La Jolla, CA, USA) was added to the culture to inhibit the activity of AR or mTOR, respectively. The concentration of these drugs in each experiment is specified in the Results section. In some experiments, the cultures were subjected to glucose deprivation. This was achieved by replacing the regular RPMI-1640 medium with a glucose-free RPMI-1640 medium from Invitrogen (Carlsbad, CA, USA).

Small interference RNA (siRNA) transfection. AR siRNA and the control scrambled siRNA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Lipofectamine was obtained from Invitrogen. Cells were transfected in 6-well plates according to product instructions. They were used for experiments 12 h after transfection.

Western blotting. The method of Western blotting used is described in a previous publication (9). Antibodies to mTOR, p70S6K, phospho-p70S6K (S371), S6, phospho-S6 (S235/236), 4EBP-1, phospho-4EBP-1 (T37/46), TSC1 and TSC2 were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies to AR, PSA and KLK2 were obtained from BD Pharmagen (San Jose, CA, USA), Lab Vision (Fremont, CA, USA), and Abcam (Cambridge, MA, USA), respectively. GADPH was used as the loading control in all Western blot analyses.

RNA isolation and quantitative real-time RT-PCR. Total RNA was isolated using the RNEASY Mini Kit from Qiagen (Valencia, CA, USA). cDNA was generated with the SuperScript VILO cDNA Synthesis Kit from Invitrogen. Reactions of quantitative real-time PCR were set up using the TagMan Universal PCR Master Mix from Applied Biosystems (Branchburg, NJ, USA). Primers for TSC2, PSA, and β-actin were obtained from Applied Biosystems. Amplification was performed on a 7900HT fast real-time PCR system from Applied Biosystems.

Cell death analysis. The analysis was performed by using a Cell Death Detection ELISA kit from Roche Applied Science (Indianapolis, IN, USA). 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, six wells of cells were used: three for the cell death assay and three for the MTT cell number assay (9). The cell death reading (measured in O.D. units) was then normalized against the MTT reading. The data were expressed as induction of cell death, *i.e.* the net increase due to treatment. Untreated cells served as the control in every experiment.

Statistical analysis. Student's *t*-test was used to determine statistical difference between treatment and control values. A p-value of <0.05 was considered significant.

Results

Bicalutamide antagonism of AR reduces mTOR activity in low testosterone. Bicalutamide is a non-steroidal anti-androgen which competitively blocks the binding of testosterone or dihydrotestosterone to AR (10). The AR in LNCaP carries a mutation, which often allows both agonists and antagonists to activate the receptor. Bicalutamide is a true anti-androgen with little potency to stimulate the mutated AR (11). The effect of bicalutamide on mTOR activity was examined in a low testosterone (0.03 nM) condition. LNCaP cells were treated with 0.5 or 1 μM bicalutamide for 15 or 24 h. Bicalutamide slightly decreased the protein level of AR, but completely blocked PSA production at both doses and time points (Figure 1A). Since PSA is a known target gene of AR, the results suggest that AR activity is depressed by bicalutamide in this experimental condition. The phosphorylation of mTOR

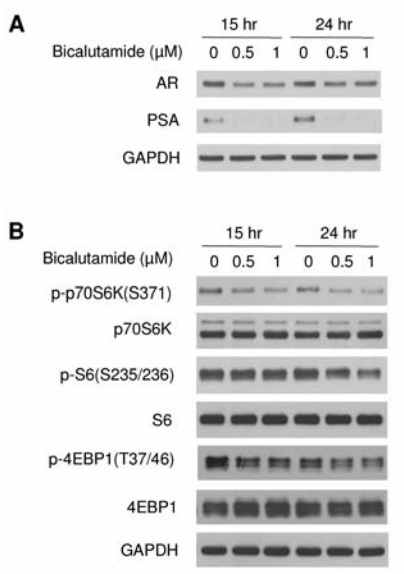

Figure 1. *Effect of bicalutamide treatment on mTOR activity. A: Bicalutamide inhibition of PSA expression as an AR target gene marker. B: Changes in the phosphorylation of mTOR downstream effectors by bicalutamide.*

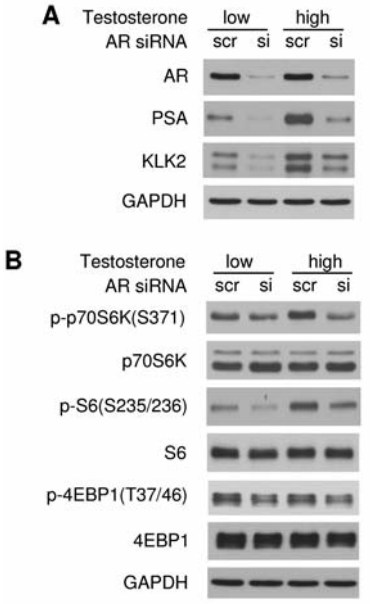

Figure 2. *Effect of AR siRNA knockdown on mTOR activity. A: Inhibition of AR target gene expression by AR siRNA. B: Changes in the phosphorylation of mTOR downstream effectors by AR siRNA knockdown.*

substrates, which include p70S6K, S6 and 4EBP-1, is used widely as an indicator of mTOR activity. Treatment with bicalutamide decreased the phosphorylation of all three mTOR substrates, although with some minor differences (Figure 1B). Phospho-70S6K and phospho-4EBP-1 were reduced roughly to a similar extent by both doses of bicalutamide and at both time points. In contrast, the decrease of phospho-S6 required a higher dose and a longer time. Total protein level of the unphosphorylated substrates was not affected by bicalutamide. The above experiment demonstrated that as little as 0.03 nM testosterone is sufficient to positively regulate mTOR activity, suggesting that mTOR is a high priority mediator of AR signaling.

AR siRNA knockdown inhibits mTOR activity in low and high testosterone conditions. In order to ascertain the contribution of an AR-dependent mechanism for the control of mTOR, the effect of AR knockdown on mTOR activity was studied in low (0.03 nM) and high (5 nM) testosterone conditions. Exposure to 5 nM testosterone is used routinely to evaluate the responsiveness of prostate cancer cells to testosterone. AR was knocked down successfully by siRNA in both testosterone conditions (Figure 2A). As expected, the expression of AR targets, such as PSA and KLK2, decreased significantly. In the scrambled siRNA control cells, the activity of AR was enhanced by adding testosterone to the culture. This observation affirmed that the low testosterone condition is insufficient to support the full function of AR.

The effect of AR knockdown on mTOR activity, as assessed by phosphorylation changes of mTOR substrates, is shown in Figure 2B. In both the low and high testosterone conditions, AR knockdown decreased the activity of mTOR. However, depending on which particular phosphorylated substrate was analyzed, there were some variations in the magnitude of the decrease. The reduction of phospho-p70S6K and phospho-S6 was greater than that of phospho-4EBP-1. By comparing the results of Figures 1 and 2, it is evident that AR knockdown produced a much more pronounced decrease of AR activity than mTOR activity. The observation suggests that AR signaling may not be the only factor controlling mTOR activity. The data of the scrambled siRNA control cells presented in Figure 2B show that the phosphorylation of p70S6K and S6 was increased by testosterone stimulation (lane 1 *vs*. lane 3). On the other hand, the phosphorylation of 4EBP-1 appeared to be much less sensitive. The reason for this discrepancy is unclear. Other factors may be involved in how mTOR differentially regulates its downstream effectors. In summary, the positive regulation of mTOR by AR was operative in low or high testosterone condition, and generally a strong AR signal would produce a more vibrant mTOR response.

AR signaling modulates the expression of mTOR regulators. TSC1 and TSC2 are negative regulators of mTOR. It is possible that the stimulation of mTOR activity might be mediated by changes in the expression of these negative

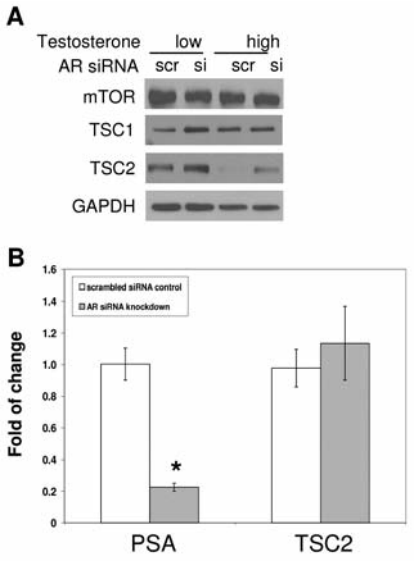

Figure 3. *AR signaling and expression of mTOR regulators. A: Effect of AR siRNA knockdown on the expression of TSC1 and TSC2. B: Effect of AR siRNA knockdown on PSA and TSC2 transcription.*

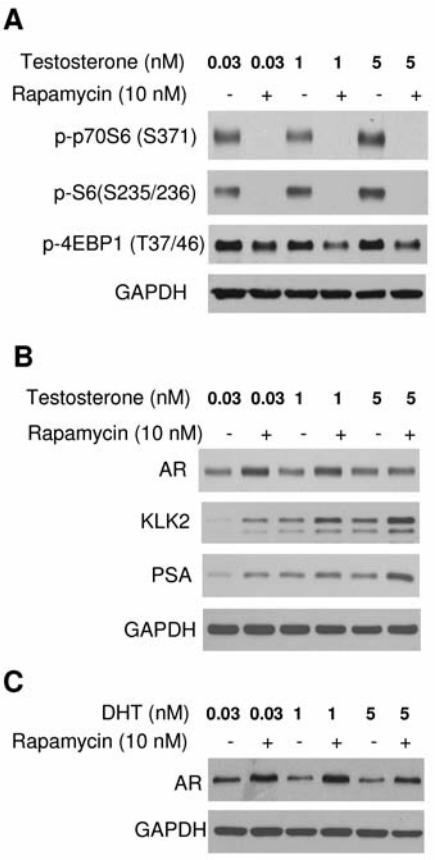

Figure 4. *Effect of mTOR inhibition by rapamycin on AR protein level and AR activity. A: Confirmation of rapamycin suppression of mTOR activity. B: Rapamycin effect on AR protein level and AR target gene expression in the presence of increasing testosterone concentrations. C: Rapamycin effect on AR protein level in the presence of increasing DHT concentrations.*

regulators. The effect of AR siRNA knockdown on mTOR, TSC1 and TSC2 was studied in both the low and high testosterone conditions. mTOR protein was not affected by any treatment condition (Figure 3A). AR knockdown increased the expression of TSC1, but only in a low testosterone condition. TSC2, on the other hand, was increased by AR knockdown in both the low and high testosterone conditions. Adding testosterone to the culture significantly decreased TSC2.

Quantitative real-time RT-PCR was carried out to determine whether AR regulates the transcription of TSC2. LNCaP cells were transfected with AR siRNA for 15 h. PSA was used as a target gene of AR to assess the successful inhibition of AR activity by AR knockdown. The results are expressed as fold of change relative to the value of the scrambled siRNA transfected control (Figure 3B). The transcription of PSA was inhibited by 70% or more due to AR knockdown. However, the transcription of TSC2 was not affected. The data thus suggest that the regulation of TSC2 by AR is not at the transcriptional level.

Rapamycin induction of AR is sensitive to testosterone. Up to this point, the results indicated that AR positively regulates mTOR activity in both low and high testosterone conditions. The next step was to address whether mTOR regulates AR in a reciprocal manner and if testosterone modulates the signal from mTOR to AR. Cells were exposed to 0.03, 1 or 5 nM testosterone, and the activity of mTOR was inhibited by treatment with rapamycin for 24 h. Rapamycin completely blocked the phosphorylation of p70S6K and S6, and decreased only marginally the phosphorylation of 4EBP-1 (Figure 4A). The inhibition of mTOR activity by rapamycin was not dependent on testosterone concentration. Rapamycin increased AR expression at 0.03 and 1 nM testosterone (Figure 4B). However, at 5 nM testosterone, the induction of AR by rapamycin was no longer evident. The data suggest that AR expression is up-regulated when mTOR is depressed, but this loop was operative only in a low testosterone condition. In cells not treated with rapamycin, raising the concentration of testosterone did not alter AR protein level, but increased the expression of PSA and KLK2. In cells treated with rapamycin, PSA and KLK2 expression was induced at all testosterone concentrations. The increase of PSA and KLK2 at 0.03 and 1 nM testosterone paralleled the increase of AR expression. However, the increase at 5 nM testosterone appeared unrelated to a change of AR level.

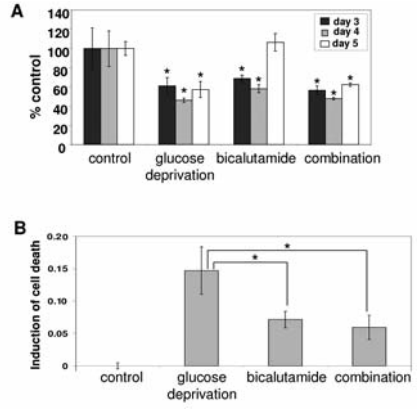

Figure 5. *Effect of concomitant glucose deprivation and bicalutamide treatment on cell growth and cell death. A: MTT cell growth data following glucose deprivation and/or bicalutamide treatment for various lengths of time. *p <0.05 compared to the corresponding control value. B: Cell death induction, as determined by the ELISA cell death assay, following glucose deprivation and/or bicalutamide treatment. *p<0.05.*

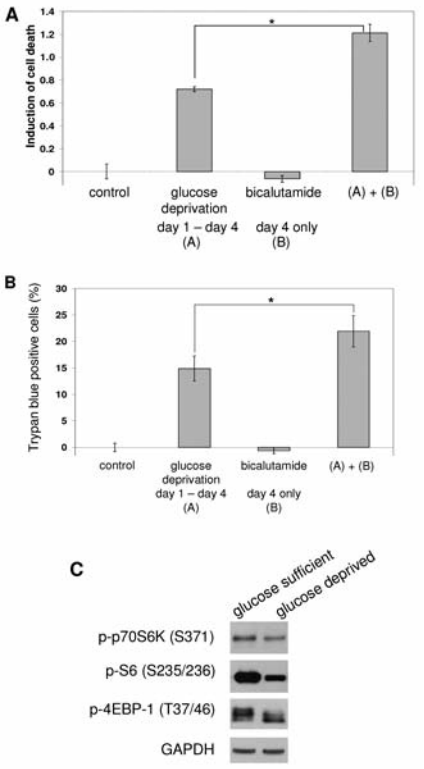

Figure 6. *Effect of sequential glucose deprivation and bicalutamide treatment on cell death. A: Cell death induction, as determined by the ELISA cell death assay, following a timed treatment schedule of glucose deprivation and/or bicalutamide. *p<0.05. B: Cell death induction as determined by the trypan blue method, same protocol as in panel A. C: Confirmation of decreased mTOR activity by glucose deprivation.*

Another experiment was carried out to determine whether dihydrotestosterone (DHT) has the same effect on rapamycin induction of AR as testosterone. LNCaP cells were treated with 10 nM rapamycin in the presence of 0.03, 1 or 5 nM DHT. In contrast to testosterone, DHT did not prevent the induction of AR by rapamycin (Figure 4C). The results suggest that testosterone and DHT may have differential effects on the cross-talk between mTOR and AR.

AR activity is critical to cell survival in low testosterone. Since mTOR is a key player in sensing and responding to nutrient deprivation, AR-mTOR signaling cross-talk may be particularly important to stress management. Cell survival is likely to be affected when AR activity is inhibited in a low nutrient condition. First, the effects of glucose deprivation, bicalutamide, or the combination treatment on cell growth were evaluated in a low testosterone condition. The MTT assay was performed after three, four or five days. Glucose deprivation inhibited cell growth by ~40-50% for the five-day duration (Figure 5A). Bicalutamide treatment for three days caused growth inhibition of ~30%. However, a longer exposure to bicalutamide actually restored growth by day five. The data suggest that the growth inhibitory effect of bicalutamide may be reversible, and cells are able to recover from growth arrest over time. The cell growth pattern in the combination-treated cells was similar to that in cells treated with glucose deprivation alone, and no further decrease in cell growth was detected with the combination compared to the individual treatments.

In order to interpret the above finding, evidence of apoptosis in the surviving cells was sought using the ELISA cell death assay after three days of treatment. This time point

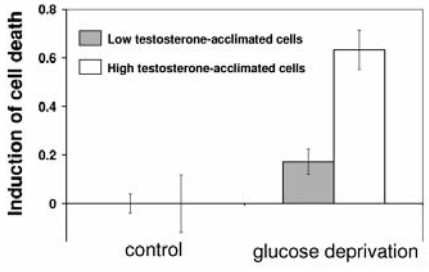

Figure 7. *Effect of glucose deprivation on induction of cell death in high testosterone- or low testosterone-acclimated cells.*

was chosen because the growth inhibitory effect of glucose deprivation was apparent on day three, while the effect of bicalutamide may already be subsiding after day three. Both glucose deprivation and bicalutamide were able to induce apoptotic cell death, although bicalutamide was less effective (Figure 5B). Apoptotic cell death in either condition was modest. The combination did not produce more apoptosis, instead, there was a decrease compared to that caused by

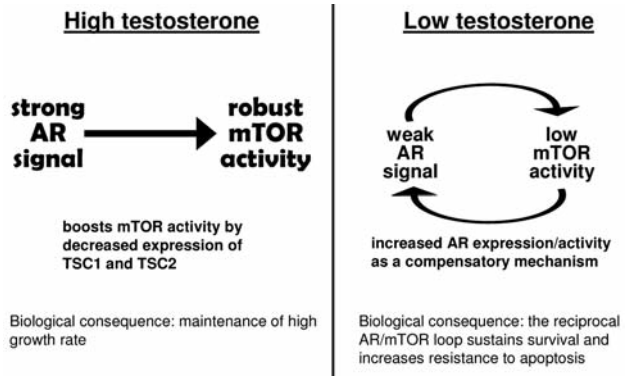

Figure 8. *A schematic representation of the AR-mTOR cross-talk in a high and low testosterone conditions.*

glucose deprivation alone. The data suggest that inhibition of AR activity may have a protective effect against glucose deprivation. However, the difference in cell response to bicalutamide or glucose deprivation should be taken into account when interpreting the data. In these experiments, glucose deprivation was achieved by incubating cells in a glucose-free medium. Cells may require time to exhaust alternative energy sources. Bicalutamide treatment, on the other hand, may have a much quicker effect on AR activity. This explanation is strengthened by the data showing that bicalutamide treatment for 24 h markedly decreased PSA expression. AR inhibition generally suppresses cell growth and slows down metabolism, thereby reducing energy demand, which may in turn lessen the sensitivity to glucose deprivation.

To test this hypothesis, an adjuvant bicalutamide protocol was designed in which cells were subjected first to glucose deprivation for three days, followed by bicalutamide treatment for another day. Bicalutamide alone was ineffective in causing apoptosis (Figure 6A). However, bicalutamide treatment following glucose deprivation produced significantly more cell death compared to glucose deprivation alone. The above experiment was performed using the ELISA cell death assay, which provides a biochemical measurement of the entire cell population. Another experiment with the same protocol was carried out, with the exception that the trypan blue method was used to asses the percentage of dead cells (Figure 6B). The trypan blue experiment produced the same pattern of cell death with the different treatments as the ELISA cell death assay. In these experiments, mTOR activity was severely diminished by glucose deprivation (Figure 6C). Thus, in the face of an energy crisis when mTOR activity is greatly compromised, AR function is needed to keep cells in a survival mode.

Resistance to glucose deprivation-induced apoptosis in low testosterone-acclimated cells. As noted above, the induction of AR protein by inhibition of mTOR activity is only operative in a low testosterone condition. Because AR is a key regulator of cell survival, an up-regulation of AR may provide additional protection against stress-related cell death. The role of testosterone in glucose deprivation-induced apoptosis was therefore studied. LNCaP cells were propagated for at least five generations in 5 nM testosterone; these were called 'high testosterone-acclimated' cells, as opposed to the 'low testosterone-acclimated' cells used in all the previous experiments. Cell death induced by glucose deprivation was assessed in both high testosterone- and low testosterone-acclimated cells after three days. The low testosterone-acclimated cells were much more resistant to apoptotic death than the high testosterone-acclimated cells (Figure 7). It can be concluded that cells which have adapted to low testosterone are much less susceptible to stress-induced death. The outcome is predictable because the low testosterone-acclimated cells are able to up-regulate AR protein and activity, and are therefore better equipped for survival in a stress situation.

Discussion

The positive regulation of mTOR by AR has been reported by Xu *et al.* (5). The authors concluded that dihydrotestosterone increases the AR-mediated transcription of a host of nutrient transporter genes, and the influx of glucose and amino acids underlies the maintenance of a robust mTOR. The results of the present study suggest a more direct way of targeting two mTOR inhibitory proteins: TSC1 and TSC2. It is not clear how AR may regulate the expression of TSC1 or TSC2. The present study was focused primarily on TSC2 because it is the more dominant regulatory signal of mTOR, while very little is known about the function of TSC1 (12). The real-time RT-PCR data showed that AR regulation of TSC2 is not at the transcriptional level. Previous work on the post-transcriptional modification of TSC2 may offer some clues to this issue. Recently, acetylation has been reported to stabilize TSC2 by preventing its degradation (13). The turnover of TSC2 is also regulated by an E3 ubiquitin ligase (14). AR may indirectly regulate TSC2 through acetylation- or ubiquitin-associated mechanisms. Another possibility may be related to the phosphorylation of TSC2, which in turn leads to its degradation (15). Since AR is known to activate multiple kinases through non-genotropic mechanisms (16, 17), inhibiting AR activity may result in increasing TSC2 stability.

The induction of AR by mTOR has been described by two other groups. Wang *et al.* (7) found that rapamycin inhibition of mTORC1 increases AR transcriptional activity *via* an Akt-dependent pathway downstream of mTORC2. Cinar *et al.* (6), on the other hand, concluded that the up-regulation of AR by rapamycin is at the translational level. An intriguing observation of the present study is that the mTOR → AR signal is sensitive only to testosterone, but not to DHT. The finding suggests that testosterone itself may serve as a modulator of intracellular signaling in addition to its role as a precursor of DHT formation.

Further investigation is needed to understand how prostate cancer cells respond to exogenous testosterone through mechanisms that are upstream, or even independent, of AR. How may cells benefit from increased AR protein? One advantage could be an enhanced sensitivity to low levels of androgen, thereby allowing cells to become resistant to ADT. Chen *et al*. (18) demonstrated that increased AR protein may amplify the output from residual ligand and alter the response to antagonist.

The findings from this study have demonstrated an intricate relationship between AR and mTOR, which is conceptualized schematically in Figure 8. What are the advantages offered by this kind of signaling interaction? When the supply of testosterone is plentiful, AR functions at full capacity and boosts mTOR activity by decreasing the expression of mTOR inhibitors. As a consequence, protein synthesis operates efficiently to support the high growth rate of cancer cells. When the supply of testosterone is scarce (*e.g*. during ADT), a weakened AR signal is responsible, at least in part, for limiting the availability of nutrients to the cells (5). Since mTOR is sensitive to nutrient levels, its activity would be diminished as a result of nutrient deprivation. The AR to mTOR connection remains intact in spite of a weakened AR signal, thereby rescuing mTOR from a crippling fate. In a sense, AR serves as a safety net to catch the freefall of mTOR in this situation. At the very least, mTOR is restored to a 'survival threshold' so that it is in a position to keep protein synthesis to a minimum. A sub-baseline mTOR level in turn stimulates AR expression in order to compensate for decreased availability of testosterone (18, 19), thus completing the loop to the mutual benefit of both partners. The reinforcement of AR function by mTOR rejuvenates the fight for survival, because the process halts what would otherwise be a downward spiral to self-destruction due to an accelerating degeneration of the AR–mTOR axis. Disrupting this loop during the window of maximal cell stress immediately after initiation of ADT may block the progression of androgen-dependent prostate cancer to ADT-resistant prostate cancer.

Acknowledgements

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