Androgens activate mitogen-activated protein kinase via epidermal growth factor receptor/insulin-like growth factor 1 receptor in the mouse PC-1 cell line

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Abstract

Androgens are the primary regulators of epididymal structure and functions. In the classical view of androgen action, binding of androgen to the intracellular androgen receptor (AR) produces the receptor-steroid complex that has high affinity for DNA response elements and regulates the transcription of target genes. In this study, we demonstrate that in epididymal cells, 5 α -dihydrotestosterone (DHT) can cause an alternative and rapid response that is independent of AR-DNA interactions and is mediated by activation of signaling pathways through the AR. We examined changes in AKT and extracellular signal-regulated protein kinases (ERK1/2) activation at early time points after DHT supplementation in the mouse proximal caput epididymis-1 cell line. DHT had no significant effect on AKT activation at any time point. However, DHT activated the ERK pathway as early as at 1 min, the pathway remained activated at 10 min,

but activation was not sustained at later time points. Interestingly, ERK activation was blocked by hydroxyflutamide (HF), indicating that early ERK activation was an AR-mediated response. DHT phosphorylates steroid receptor co-activator (SRC) kinase, and this activation was required for the ERK response. EGFR and IGF1R were downstream of SRC, and these two receptors together contributed to enhance ERK and cAMP response element-binding protein (CREB) phosphorylation. We postulate that this rapid action of androgen may ultimately act to modulate the transcription of genes regulated by AR in the nucleus. These results support the hypothesis that DHT can activate a pathway involving the sequential activation of MEK, ERK1/2, and CREB through the EGFR/IGF1R in an epididymal cell line.

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Introduction

The epididymis plays an important role in male fertility, because it is the site where spermatozoa are matured and stored. Maturation of spermatozoa, including the acquisition of motility and capability to fertilize an oocyte, occurs during transit through the unique and dynamic microenvironment of the epididymal lumen. The specificity of the microenvironment is primarily under the influence of androgens that ultimately regulate epididymal cell structure and function (Orgebin-Crist 1967, Orgebin-Crist & Tichenor 1973, Turner *et al.* 1984, Robaire & Viger 1995). Principal cells are the major cell type of this tissue (Trasler *et al.* 1988) and are particularly sensitive to androgen removal (Moore & Bedford 1979a,b). The major circulating androgen testosterone is metabolized by 5 α -reductases into the potent androgen 5 α -dihydrotestosterone (DHT; Gloyna & Wilson 1969, Monsalve & Blaquier 1977, Robaire *et al.* 1977). Androgens mediate their effects by diffusing through the plasma membrane and binding to intracellular androgen receptors

(ARs). The AR functions as a ligand-inducible transcription factor modulating transcription of target genes (He *et al.* 1999, Heinlein & Chang 2002). In addition to this slow genomic action of androgens, several lines of evidence suggest that androgens, like progestins and estrogens, can exert rapid non-genomic effects (Christian *et al.* 2000, Falkenstein *et al.* 2000).

Unlike genomic effects that take hours or days to exert their action, non-genomic effects are activated within seconds or minutes (Sachs & Leipheimer 1988, Crews & Erikson 1993, Wehling 1997, Revelli *et al.* 1998, Christian *et al.* 2000, Falkenstein *et al.* 2000). The extracellular signal-regulated protein kinases (ERK1/2), members of the MAPK superfamily, are activated in response to androgens. These protein kinases play a key role in cell growth, differentiation, and function at both the transcriptional and the post-transcriptional levels, by phosphorylating a range of proteins, including nuclear transcription factors, cytoskeletal proteins, other protein kinases, and receptors for hormones and growth factors (Crews & Erikson 1993, Nguyen *et al.* 2005, Gatson *et al.* 2006). Androgens can also activate the

phosphatidylinositol-3 kinase (PI-3K) and AKT pathway rapidly in a ligand binding-independent manner (Lutz *et al.* 2003, Sun *et al.* 2003, Kang *et al.* 2004). However, it is unknown whether those two pathways, ERK1/2 and AKT, are activated in epididymal cells in response to DHT.

Studies have indicated that the non-genomic action of androgens depends on the ability of the AR to interact with the steroid receptor co-activator (SRC; Heinlein & Chang 2002); activation of PI-3K by SRC family kinases has also been established (Liu *et al.* 1993). The androgen-AR complex interacts with the SH3 domain of SRC kinase and leads to a rapid activation of RAF-1 and MEK, and as a result, the ERK pathway; thus, the SRC-MAPK is one of the major routes in signal transduction (Migliaccio *et al.* 2000, Kousteni *et al.* 2001, Castoria *et al.* 2004, Cheng *et al.* 2007). Furthermore, SRC is also able to activate EGFR directly or indirectly and subsequently the MAPK pathway by phosphorylating tyrosine residues on the cytoplasmic face of EGFR (Stover *et al.* 1995); however, the role of IGF1R in mediating the rapid action of androgen through ERK pathway activation remains to be elucidated. Activation of SRC by androgen occurs only in the cell membrane, without the interaction of EGFR with AR, whereas EGFR, estrogen receptor, and SRC make a complex upon estrogen stimulation (Hitsugi *et al.* 2007).

In Sertoli cells, the rapid action of androgen leads to activation of transcription factors such as cAMP response element-binding protein (CREB); the latter is an essential factor required for Sertoli cells to support spermatogenesis (Fix *et al.* 2004, Cheng *et al.* 2007). Similarly, studies have shown that androgens upregulate the IGF1R by inducing CREB activation through activation of the c-SRC/ERK pathway in prostate cancer cells (Pandini *et al.* 2005, Genua *et al.* 2009), indicating the role of non-genomic action of androgens in regulation of gene transcription.

While several studies have established different pathways mediated by a non-genomic action of androgens, in this study we present an integrated model of an extranuclear, AR-mediated, rapid MAPK activation pathway that acts through the activation of EGFR/IGF1R signaling in an epididymal cell line.

Materials and Methods

Cell culture and treatments

The mouse proximal caput epididymis-1 (PC-1) cell line (kindly provided by Dr M-C Orgebin-Crist, Vanderbilt University) was derived from primary culture of epididymal cells from transgenic mice harboring a temperature-sensitive simian virus 40 large T antigen. It is a pure population of epithelium-derived caput principal cells (Araki *et al.* 2002). PC-1 cells were grown in Iscove's Modified Dulbecco's Medium (without phenol red) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 4 mM glutamine,

0.7% penicillin-streptomycin (25 000 U penicillin sodium and 25 mg streptomycin sulfate), and 1 nM 5 α -DHT. All cell culture products were purchased from Wisent (St Bruno, QC, Canada), and DHT was from Steraloids, Inc. (Newport, RI, USA). PC-1 cells were cultured at 33 °C with 5% CO₂. FBS was replaced with charcoal-filtered FBS two passages before the experimental procedure, and the latter was used for the duration of the experiments. The cells were seeded onto flasks for 2, 4, and 6 d in the media as described above and subjected to vehicle as control or to different DHT concentration. Media were changed every 24 h. Viability of the cells was determined by using trypan blue. Total RNA of the cells was then extracted. To assess the signaling activation, cells were seeded for 2 d in the media as described above. Proteins from the cells were extracted at various time points after changing the media. In some cases, cells were pretreated for 30 min with the signaling pathway inhibitors prior to being stimulated with DHT for 5 min.

Inhibitors employed included hydroxyflutamide (HF; Toronto Research Chemicals, Inc., Toronto, ON, Canada), tyrphostin I-OMe-AG 538 (T7697, Sigma), tyrphostin AG 1478 (9842, Cell Signaling Technology, Beverly, MA, USA), and LY294002, PD98059 and PP2 were obtained from EMD Biochemicals (San Diego, CA, USA).

RNA extraction and DNase treatment

Each experimental group comprised five different replicates. Total RNA was extracted and DNase treated using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. RNA concentrations were assessed by OD determination at 260 nm (DU7 spectrophotometer, Beckman, Montreal, QC, Canada). The RNA used for microarray analysis had an A260/A280 ratio > 1.8. To verify the quality of each sample, 2 μ g RNA were run on a denaturing gel containing 1% agarose.

Quantitative real-time PCR

Quantitative real-time PCR was done using the LightCycler system (Roche Diagnostics) with the QuantiTect One-Step SYBR Green RT-PCR kit (Qiagen) according to the manufacturer's protocol. The specific primer sequences for *Ar*, *Igf-1*, and *cyclophilin A* (*Ppia*) were designed using Primer3 software (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/>). *Cyclophilin A* was chosen as the endogenous control as its mRNA levels did not change with androgen manipulation (Palladino & Hinton 1994). The primer sequences are as follows: *cyclophilin A*: forward primer 5'-GTGGTCTTTGGGAAGGTGAA-3', reverse primer 5'-GTTGTCCACAGTCGGAGATG-3' (NM_008907); *Ar*: forward primer 5'-CAGGCAAAAGCACTGAAGAGA-3', reverse primer 5'-GAGCCAGCGGAAAGTTGTAG-3' (NM_013476); *Igf-1*: forward primer 5'-GGCATTGTG-GATGAGTGTG-3', reverse primer 5'-GTCTTGGG-CATGTCAGTGTG-3' (NM_178866). The PCR products

were confirmed to be of appropriate size (128 for *Igf-1* and 216 for *Ar*). The specificity of the qRT-PCR primers was confirmed by melting curve analysis. Derivatives of the melting curves contained a single peak, indicating that a single PCR product has been generated; no primer-dimers were observed. All standards and samples were assayed in duplicate. Average values for the target gene RNA concentrations of each sample were normalized to the average value for *cyclophilin* RNA concentration in the same sample.

Western blot analysis

Whole-cell extracts for each group were collected into lysis buffer containing protease and phosphatase inhibitors using the cellular fractionation kit (Cat. No: 40010) from Active Motif (Carlsbad, CA, USA), according to the manufacturer's protocol. We evaluated total protein concentration by the Bradford method, using the Bio-Rad protein assay (Bio-Rad Laboratories). Protein samples (15 µg per group) were boiled with loading buffer for 5 min and fractionated by SDS-PAGE using 10% acrylamide gels. Prestained precision standards (Bio-Rad Laboratories) were used as molecular weight markers. The fractionated proteins were transferred to a Hybond-P membrane (Amersham Biosciences UK). They were incubated with primary antibodies (1:1000; unless stated otherwise), followed by HRP-conjugated second antibodies (1:10 000; Amersham Pharmacia Biotech UK).

The primary antibodies employed were against total MAP kinase 1/2 (ERK 1/2), CREB, and phospho-CREB (Ser¹³³) (nos 06-182, 06-863, and 06-519, Millipore Corporation, Temecula, CA, USA), phospho-44/42 MAPK (ERK1/2) (Thr²⁰²/Tyr²⁰⁴), total AKT (pan), and phospho-AKT (Ser⁴⁷³) (nos 9106, 4691, and 4060, Cell Signaling Technology), a polyclonal antibody against phospho-SRC [pY⁴¹⁸] (44660G, Invitrogen Corporation), a monoclonal antibody against c-SRC (B-12) (sc-8056, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The levels of P-ERK, P-AKT, P-SRC, and P-CREB were normalized to overall ERK, AKT, SRC, and CREB expression levels respectively. Actin (1:4000, sc-1616) was used as second loading control and was detected using a secondary, donkey anti-goat IgG HRP antibody (1:10 000, sc-2056). Western blots were visualized with the Enhanced Chemiluminescence Plus kit and Hyperfilm-Enhanced Chemiluminescence (Amersham Biosciences UK). Quantification of western blot data was done by line densitometry using a ChemiImager 4000 imaging system with AlphaEase (version 5.5 software; Alpha Innotech, San Leandro, CA, USA).

Statistical analysis

Statistical analysis was done using *t*-test or one-way ANOVA followed by the multiple comparisons Dunnett's test. Data were expressed as mean ± S.E.M. Values of $P \leq 0.05$ were regarded as statistically significant.

Results

Cell viability is not affected by treatment

The effects of androgen withdrawal and treatment on cell viability were determined using the trypan blue staining technique. Cells did proliferate; the number of cells had doubled after 2 d and was increasing more slowly after 4 d (Supplementary Figure 1, see section on supplementary data given at the end of this article). Very few dead cells were observed within 6 days, although significant cell death was observed by 8 d after androgen withdrawal (data not shown).

Ar and *Igf1* genes are expressed in PC-1 cell line

The mRNA expression of *Ar* and *Igf1* genes was determined by qRT-PCR at 2, 4, and 6 d in androgen-deprived cells before and after DHT treatment. DHT had no significant effect on *Ar* expression in cells compared with that in vehicle-treated cells (Supplementary Figure 2, see section on supplementary data given at the end of this article).

Figure 1 Effect of DHT on AKT activation. Cells were treated with vehicle (white bar) or DHT (0.1 nM hatched bar, 1 nM grey bar, and 10 nM black bar) for 10, 30, or 45 min. AKT phosphorylation was analyzed by western blot. Each bar represents the mean of five replicates ± S.E.M.

In comparison, *Igf1* expression was significantly increased at 2, 4, and 6 days in DHT-treated cells compared with the control (Supplementary Figure 2, see section on supplementary data given at the end of this article).

Activation of AKT and ERK1/2 by DHT

To assess whether DHT can enhance the activation of the AKT or the ERK pathway, we stimulated cells with DHT (0.1, 1, or 10 nM) for 10, 30, or 45 min. The amounts of phosphorylated AKT and ERK were measured relative to total AKT and ERK. DHT had no significant effect on AKT phosphorylation compared with control at any time point (Fig. 1). In contrast, DHT (0.1 and 1 nM) significantly increased ERK phosphorylation at 10 min; this activation was not sustained at 30 and 45 min (Fig. 2a). To determine whether this activation occurred earlier than 10 min, ERK activation by DHT (0.1 and 1 nM) was investigated at 1, 3, and 5 min. DHT significantly increased ERK phosphorylation levels compared with control at all time points (Fig. 2b). Similarly, AKT activation in response to DHT was also measured at earlier and later time points,

but no significant changes were observed (data not shown). DHT did not alter total expression of AKT or ERK relative to actin.

ERK1/2 is activated by DHT via an AR-mediated mechanism

To determine the selectivity of DHT in stimulating ERK through an AR-mediated mechanism, cells were pretreated with HF, a non-steroidal and reversible AR antagonist, before stimulation with DHT (0.1, 1, or 10 nM). In order to see the complete effect of HF, the dose chosen was 1000–10 000 times higher concentration than that of DHT (Yamamoto *et al.* 2000, Urushibara *et al.* 2007). Pretreatment with HF repressed ERK phosphorylation significantly in DHT-treated cells compared with control. In cells without HF, treatment with 1 nM DHT increased p-ERK compared with control cells without HF, which is similar to the ratio between cells treated with and without 1 nM DHT in the presence of HF that could be due to the blocking effect of HF on low endogenous androgen in cells or non-specific inhibitory effect of HF on cells. Interestingly, the repression of ERK phosphorylation by HF was partially reversed at higher DHT concentrations (Fig. 3).

Figure 2 DHT elicits an increase in phospho-ERK1/2. (a) Cells were treated with vehicle (white bar) or DHT (0.1 nM, 1 nM, and 10 nM black bar) for 10, 30, or 45 min. (b) Cells were treated with vehicle (white bar) or DHT (0.1 nM and 1 nM hatched bar) for 1, 3, 5, or 15 min. ERK phosphorylation was analyzed by western blot. (*) Indicates that values were significantly different from vehicle-treated controls ($P \leq 0.05$). Each bar represents the mean of five replicates $\pm$ S.E.M.

Figure 3 Hydroxyflutamide (HF) blocks DHT-induced ERK1/2 phosphorylation. Cells were pretreated for 30 min with hydroxyflutamide (10 μ M) before stimulation for 5 min with vehicle (white bar) or DHT (0.1 μ M, 1 μ M, and 10 μ M black bar). ERK phosphorylation is shown by western blot. (*) Indicates that P-ERK was significantly repressed by HF compared with control, and this effect was reversed by increasing the concentration of DHT ($P \leq 0.05$). Each bar represents the mean of five replicates $\pm$ S.E.M.

SRC kinase is phosphorylated by DHT and is required for ERK1/2 activation

We investigated the activation of c-SRC by DHT and found that DHT (1 nM) significantly increased SRC phosphorylation compared with control at 1 (data not shown) and 5 min (Fig. 4). Although it is possible that the activity of other SRC family members may be regulated by DHT, this result indicates that androgen stimulation results in a rapid phosphorylation of SRC in PC-1 cells. To assess whether SRC activation was required for DHT-mediated induction of ERK activation, cells were pretreated with PP2 before stimulation with DHT (1 nM). ERK phosphorylation was significantly repressed by inhibition of SRC activation (Fig. 5). The inhibition of ERK phosphorylation by PP2, a

SRC specific inhibitor, was comparable to that of cells pretreated with MEK inhibitor, PD98059. In addition, ERK activation was significantly repressed by PI-3K inhibitor, LY294002 (Fig. 5). These results suggest that DHT-dependent activation of ERK requires the activated SRC kinase, as well as PI-3K and MEK, the kinase that phosphorylates ERK. P-ERK was not significantly decreased with the lower concentration of inhibitors (2.5 μ M LY and PD, and 0.5 μ M PP2; data not shown).

DHT activates the ERK pathway via IGF1R and EGFR

To determine whether ERK is activated by DHT via IGF1R and EGFR, cells were pretreated with IGF1R inhibitor (AG1024), EGFR inhibitor (AG1478), and the combination of both inhibitors before stimulation with DHT (1 nM). Increased ERK phosphorylation by DHT was significantly repressed by 25 μ M IGF1R inhibitor or 1 μ M EGFR inhibitor. Interestingly, using lower concentrations of the two inhibitors in combination, 10 μ M IGF1R inhibitor and 50 nM EGFR inhibitor, ERK activation was significantly blocked, although at these concentrations each alone had no effect on the level of P-ERK (Fig. 6). These results suggest

Figure 4 DHT induces SRC kinase activation. Cells were treated with vehicle (white bar) or DHT (0.1 μ M, 1 μ M, and 10 μ M black bar) for 5 min. Relative expression of SRC phosphorylation was analyzed by western blot. DHT (1 nM) significantly increased P-SRC compared with control ($*P \leq 0.05$). Each bar represents the mean of four replicates $\pm$ S.E.M.

Figure 5 DHT-mediated induction of ERK1/2 requires SRC kinase. Cells were pretreated for 30 min with SRC inhibitor PP2 (1, 5, and 10 μ M), MEK inhibitor PD98059 (5, 10, and 20 μ M), or PI-3K inhibitor LY294002 (5, 10, and 25 μ M) before stimulation for 5 min with vehicle (white bar) or DHT (0.1 μ M and 1 nM). ERK phosphorylation was analyzed by western blot. The letter b indicates that values were significantly different ($P \leq 0.05$) compared with control value (a). Each bar represents the mean of four replicates $\pm$ S.E.M.

that DHT mediates induction of ERK activation via IGF1R and EGFR and indicate that there is crosstalk between these two receptors.

SRC acts upstream of IGF1R and EGFR

To test whether the SRC kinase required for ERK phosphorylation was acting upstream of IGF1R and EGFR, cells were pretreated with inhibitors of IGF1R and EGFR alone or a combination of the two inhibitors prior to stimulation with DHT (1 nM), and then SRC activation was assessed. The latter was not affected by IGF1R and EGFR inhibitors at any concentrations that repressed ERK phosphorylation (Supplementary Figure 3, see section on supplementary data given at the end of this article), suggesting that IGF1R and EGFR act downstream of SRC.

Activation of ERK1/2 by DHT leads to CREB phosphorylation

To ensure that increased ERK phosphorylation resulted in an increased activation of CREB, we tested whether there was an increase in the phosphorylation of CREB as a result of treatment with DHT and, if so, whether this increase was repressed by ERK inhibition. Cells were treated with vehicle or DHT (0.1 or 1 nM) or were pretreated with a

combination of IGF1R and EGFR inhibitors, before stimulation with DHT; P-CREB was assessed. DHT (1 nM) significantly increased CREB phosphorylation (Fig. 7a), but this activation was partially repressed in cells pretreated with IGF1R and EGFR inhibitors (Fig. 7b). These results suggest that the activated ERK can stimulate transcription factors such as CREB.

Discussion

The results from these studies suggest that there is a mechanism for androgen action in epididymal epithelial cells that utilizes rapid activation of the MAP kinase pathway via IGF1R and EGFR and activation of CREB transcription factor. DHT increased IGF1 expression compared with the control, without having a mitogenic effect in the epididymal cells or killing them. Similar results for ERK activation have been observed in studies of breast cancer and prostate cancer cells, in which androgen activates the ERK pathway within minutes (Peterziel *et al.* 1999, Zhu *et al.* 1999). Androgen-induced phosphorylation of ERK1/2 occurred within 1 min

Figure 6 IGF-1R and EGFR inhibitors block P-ERK1/2. Cells were pretreated for 30 min with tyrphostin I-OMe-AG 538 (5, 10, and 25 μ M) or tyrphostin AG 1478 (25, 50, 150 nM, and 1 μ M) before stimulation for 5 min with vehicle (white bar) or DHT (1 nM). ERK phosphorylation was analyzed by western blot. The letter b indicates that values were significantly different ($P \leq 0.05$) compared with control values (a). Each bar represents the mean of five replicates $\pm$ S.E.M.

Figure 7 DHT induces CREB activation. (a) Cells were treated with vehicle (white bar) or DHT (0.1 ▨ and 1 nM ▩) for 5 min. CREB phosphorylation was analyzed by western blot. DHT (1 nM) significantly increased P-CREB compared with control (* $P \leq 0.05$). Each bar represents the mean of four replicates $\pm$ S.E.M. (b) Cells were pretreated for 30 min with IGF1R and EGFR inhibitors before stimulation for 5 min with DHT (1 nM) for 5 min. Inhibitors partially repressed P-CREB. The figure shown is representative of three experiments.

of androgen stimulation and was sustained for 10 min. However, AKT phosphorylation was not altered upon androgen administration. Activation of ERK within minutes was repressed by reducing the activity of the AR, suggesting that this action occurred through an AR and was not

mediated via binding of their cognate nuclear receptors to DNA, but rather through direct stimulation of cell signaling pathways. A schematic representation of the proposed pathway described in these studies is presented in Fig. 8.

The mechanism by which the androgen-AR complex initiates signaling in epididymal cells is not known. Recently, a population of AR has been localized to the plasma membrane in Sertoli cells, where testosterone rapidly and transiently promotes AR localization to the plasma membrane (Cheng *et al.* 2007). AR has also been localized to the plasma membrane in *Xenopus* oocytes and in hypothalamic cell lines (Shakil *et al.* 2002, Lutz *et al.* 2003). Association of AR with caveolin-containing membrane fractions is believed to facilitate interactions with their cognate ligands and shift the complex near membrane-associated signaling molecules, such as SRC kinase, PI-3K, or G proteins (Okamoto *et al.* 1998, Lu *et al.* 2001).

The non-receptor tyrosine kinase SRC is a major factor in signal transduction (Song *et al.* 2002, Razandi *et al.* 2003, Unni *et al.* 2004). We have shown that SRC is activated by DHT at 5 min, and that it is required for ERK activation. The essential role of SRC kinase in the non-genomic action of ARs was demonstrated in studies on embryonic fibroblasts derived from SRC $^{-/-}$ mice in which the cells do not show rapid activation of the MAPK pathway in response to ligand binding to AR (Kousteni *et al.* 2001). In LNCaP prostate cells, androgen triggers the direct association of the proline-rich region of AR and the SH3 domain of SRC, causing the activation of SRC (Migliaccio *et al.* 2000); the latter is known

Figure 8 Signaling pathway activated by DHT in epididymal epithelial cells. DHT bound to the AR can activate a linear pathway involving the sequential activation of SRC, MEK, ERK1/2, and CREB through the EGFR/IGF1R in epididymal epithelial cells.

to rapidly induce the formation of Shc/Grb2/SOS complexes, leading to the activation of RAS and subsequently stimulation of a RAF MAPK kinase kinase, the initial member of the MAPK pathway (Rusanescu *et al.* 1995). Activation of PI-3K by SRC kinase has been shown (Liu *et al.* 1993). Our data indicate that inhibition of PI-3K significantly reduces ERK1/2 activation.

The IGF and EGF ligands are mitogenic for both normal and tumorigenic mammary epithelial cells *in vitro* (Rosfjord & Dickson 1999). The IGF1 and EGF receptors share a common molecular structure, with a cytoplasmic tail containing tyrosine residues; activation of these receptors ultimately leads to the activation of the PI-3K and MAPK signal transduction pathways (Baserga *et al.* 1997, Jorissen *et al.* 2003, Kolch 2005). SRC is capable of directly activating EGFR by phosphorylating tyrosine residues on the cytoplasmic face of EGFR and subsequently the MAPK pathway (Stover *et al.* 1995, Biscardi *et al.* 1999). Our finding identified the essential role of IGF1 and EGF receptors in DHT-induced ERK1/2 activation in epididymal epithelial cells. Using a combination of specific IGF1R and EGFR inhibitors, we observed a synergistic effect on blocking DHT-mediated phosphorylation of ERK. This effect further led to an increased phosphorylation of CREB transcription factor and presumably gene expression stimulation. CREB bound to cAMP response element motifs (TGACGTA) (Montminy *et al.* 1986, Benbrook & Jones 1990) in gene promoters is able to associate with the binding protein coactivator that facilitates the recruitment of RNA polymerase to the transcription initiation site (Meyer & Habener 1993, Kwok *et al.* 1994). For instance, CREB phosphorylation has been linked to the activation of numerous Sertoli cell genes that contribute to germ cell development and survival (Scobey *et al.* 2001), and upregulation of IGF1R in prostate cancer cells (Genua *et al.* 2009).

Taken together, these data identify a rapid signaling pathway that is activated by DHT stimulation in an epididymal epithelial cell line. This represents the first study in cells of the epididymis to report the upstream signaling components for ERK activation through the AR. While the results from this study demonstrate potential rapid signaling mechanisms by DHT in an epididymal cell line, further studies are required to establish the role of this pathway *in vivo*. Targeting this signaling pathway should be considered as a means of regulating androgen action, and hence function, in this tissue.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-10-0223>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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