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Original Articles

SMILE upregulated by metformin inhibits the function of androgen receptor in prostate cancer cells

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ABSTRACT

Metformin, a diabetes drug, has been reported to inhibit the growth of prostate cancer cells. In this study, we investigated the effect and action mechanism of metformin on the function of androgen receptor (AR), a key molecule in the proliferation of prostate cancer cells. Metformin was found to reduce androgen-dependent cell growth and the expression of AR target genes by inhibiting AR function in prostate cancer cells such as LNCaP and C4-2 cells. Interestingly, metformin upregulated the protein level of small heterodimer partner-interacting leucine zipper (SMILE), a coregulator of nuclear receptors, and knockdown of SMILE expression with shRNA abolished the inhibitory effect of metformin on AR function. Further studies revealed that SMILE protein itself suppressed the transactivation of AR, and its ectopic expression resulted in the repressed expression of endogenous AR target genes, PSA and NKX3.1, in LNCaP cells. In addition, SMILE protein physically interacted with AR and competed with the AR coactivator SRC-1 to modulate AR transactivation. As expected, SMILE repressed androgen-dependent growth of LNCaP and C4-2 cells. Taken together, these results suggest that SMILE, which is induced by metformin, functions as a novel AR corepressor and may mediate the inhibitory effect of metformin on androgen-dependent growth of prostate cancer cells.

Introduction

Prostate cancer is the most commonly diagnosed malignancy in men, representing one third of all new cancer cases each year in the United States. Development and progression of prostate cancer are dependent upon the function of androgen receptor (AR) [1,2], and early-stage prostate cancer is sensitive to androgens, such as testosterone and dihydrotestosterone (DHT). Therefore, the first-line treatment for disease is designed for specific inhibition of AR activity, mostly through either depletion of ligand and/or use of AR antagonists [3–5]. Such intervention strategies are highly effective in the induction of cell death in early-stage AR-dependent prostate cancer cells [6,7]. However, recurrent tumors ultimately arise approximately a couple of years later of the treatment. Analyses of recurrent tumors and recapitulation of therapeutic resistance in model systems strongly suggest that aberrant AR reactivation is responsible for tumor recurrence [4]. Given the importance of AR activity in prostate cancer development, growth, and progression, efforts have been made to delineate factors involved in the regulation of AR activity.

AR belongs to the steroid receptor subclass of nuclear receptors [8–10]. Like other nuclear receptors, AR protein is comprised of three functional domains: an N-terminal domain (NTD) with the principle transcriptional activation function (AF-1), a central DNA-binding and hinge domain (DBD-h), and a C-terminal ligand-binding domain with another transcriptional activation function domain (AF-2) [11,12]. AR is activated by ligand binding, which causes dissociation of the receptor from heat-shock proteins and translocation to the nucleus [13,14]. Active AR homodimers recognize and bind specific DNA sequences, called androgen-response elements (AREs), which are located in the promoter and/or enhancer region of androgen-responsive genes [1]. Subsequent recruitment of coregulators controls the ability of AR to stimulate transcription of target genes [15,16].

Metformin, a widely used antidiabetic drug, belongs to the biguanide class of oral hypoglycemic agents. While metformin is used primarily in the treatment of type II diabetes, its beneficial effects have also been observed in the treatment of polycystic ovarian syndrome [17], nonalcoholic fatty liver disease [18] and premature puberty [19]. Of particular importance is that recent evidence has pointed out that metformin might reduce the risk of cancer [20]. In particular, metformin has been shown to reduce the risk of

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prostate cancer in Caucasians [21–23]. It suppressed cyclin D1 expression and pRb phosphorylation in an AMP-activated protein kinase (AMPK) independent manner [24], and combination of metformin and 2-deoxyglucose induced p53-dependent apoptosis in prostate cancer cells [25]. In addition, metformin-mediated apoptosis was elevated by blockade of MAPK signaling pathway in prostate cancer cells [26]. However, the cellular mode of action and cellular targets of metformin in cancer cells are poorly understood, although the mechanism of action of metformin has been well studied in liver, adipose tissue, and heart.

SMILE, previously referred to Zhangfei (ZF), has been identified as a protein that interacts with herpes simplex virus-related host cell factor 1 (HCF-1), and has been proposed to play a role in inhibiting the replication of herpes simplex virus [27,28]. SMILE harbors a conserved basic region leucine-zipper (bZIP) domain, which is significantly similar to that of other CREB/ATF bZIP family members. Although SMILE can homodimerize like other bZIP proteins, it lacks the ability to bind to DNA as a homodimer [28,29]. SMILE has recently been shown to augment the DNA binding ability of ATF4 to the cAMP response element (CRE) through an association with ATF4 [30]. In addition, SMILE has been reported to function as a coregulator of estrogen receptor (ER) signaling and a corepressor of the glucocorticoid receptor (GR), constitutive androstane receptor (CAR), hepatocyte nuclear factor 4α (HNF4α), and estrogen receptor-related receptor γ (ERRγ) [31–33]. However, the detailed role of SMILE in the function of other nuclear receptors still needs to be clarified.

In the present study, we investigated the molecular mechanism by which metformin, a diabetes drug reported to be effective for the treatment of prostate cancers, suppressed the function of AR in prostate cancer cells. Metformin upregulated the protein level of SMILE, and subsequently, SMILE inhibited AR transcriptional activity in prostate cancer cells. We further demonstrated direct interaction of SMILE with AR, and its competition with AR coactivator SRC-1 in the modulation of AR transactivation. Finally, SMILE overexpression resulted in the suppression of androgen-dependent proliferative activity of LNCaP and C4-2 cells. These findings may provide a new role of metformin-induced SMILE in prostate cancer cells and suggest a target molecule for prostate cancer treatment.

Materials and methods

Plasmids

Mammalian expression plasmids of mouse AR, SRC-1, ARE2-TATA-Luc, PSA-Luc, and MMTV-Luc, and bacterial expression plasmids of GST-AR deletion mutants have been previously described [34]. Bacterial expression plasmids of GST-SMILE mutants, mammalian expression vectors of human SMILE and its deletion mutants, pEGFP-SMILE, pSUPER-shSMILE#1 (US; non-specific for SMILE), pSUPER-shSMILE#2 (shSMILE) and adenovirus of GFP, SMILE, and dominant-negative AMPK (DN-AMPK) have also been previously described [31–33,35].

Cell culture and transient transfection assay

PPC-1 and HEK293 human cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Hyclone) supplemented with 10% fetal bovine serum (FBS). LNCaP cells were purchased from the American Type Culture Collection (ATCC CRL-1740). The LNCaP and C4-2 human prostate cancer cells were maintained in RPMI 1640 (Hyclone) supplemented with 5% FBS.

Twenty-four hours prior to transfection, cells were seeded into 24-well plates in the culture medium supplemented with 5% charcoal-stripped FBS. Transfections were performed using SuperFect (Qiagen) transfection reagent for HEK293T cells, and Lipofectamine 2000 (Invitrogen) transfection reagent for PPC-1 and LNCaP cells according to the manufacturers' instructions. Cells were transfected with expression vectors, a reporter gene, and the control β-galactosidase expression plasmid pCMVβ (Clontech). Total DNA amount was kept constant by addition of the pcDNA3 empty vector. After 24 h of transfection, cells were treated with 10 nM DHT in the presence or absence of 5 mM metformin. After a 24 h incubation period, cells were lysed using lysis buffer containing 0.1% Triton X-100 and 0.2 M Tris-HCl (pH 8.0). Luciferase and β-galactosidase activities were assayed as previously described [36]. The level of luciferase activity was normalized to that of β-gal expression.

Northern blot analysis and qRT-PCR

Total RNA was prepared from LNCaP cells using Tri reagent (Molecular Research Center Inc). Twenty micrograms of total RNA was separated on a 1.2% denaturing agarose gel, transferred to a Zeta probe nylon membrane (Bio-Rad), and immobilized by ultraviolet cross-linking. The membrane was hybridized with random-primed α-^{32}P-labeled PSA and NKX3.1 cDNA probes as previously described [34]. The membrane was reprobed for actin as a loading control.

For qRT-PCR, two microgram of total RNA was used for RT reaction with M-MLV Reverse Transcriptase according to the manufacture's manual (Promega). The cDNA was used to perform qRT-PCR using specific primers for PSA (forward: 5'-GGCCAGGTATTTCAGGTCAG-3' and reverse: 5'-CCACGATGGTGTCCTTGATC-3'), NKX3.1 (forward: 5'-TTCCTCATCCAGGACATCCT-3' and reverse: 5'-GTCTTATAGCGTCT GTTCTGGA-3'), and β-actin (forward: 5'-GAGACCTTCAACACCCCAGCC-3' and reverse: 5'-CCGTCAGGCAGCTCATAGCTC-3'). Expression levels of PSA and NKX3.1 were normalized to that of β-actin.

Western blot analysis

Cells were harvested with RIPA cell lysis buffer (50 mM Tris-HCl at pH 8.0, 100 mM NaCl, 1% NP-40, 5 mM EDTA at pH 8.0, 1 μg/ml aprotinin, 0.1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.1 mM phenyl-methylsulphonyl fluoride). Total protein (50–120 μg) was separated by SDS-PAGE and transferred to a nitrocellulose membrane (Sigma-Aldrich). Western blot analyses were performed with anti-AR, anti-PSA, anti-Tubulin, anti-Lamin B, anti-Actin, anti-GFP (Santa Cruz Biotechnology), anti-phospho-AMPK, anti-AMPK (Cell Signaling Technology), anti-GAPDH (Epitomics), and anti-Zhangfei (SMILE) (Abcam) antibodies. The signals were then detected with an ECL kit (Amersham Pharmacia).

Coimmunoprecipitation

HEK293T cells were transfected with indicated mammalian expression plasmids and starved in DMEM containing 5% charcoal-stripped FBS for 24 h. Cells were then treated with 10 nM DHT for 24 h, and harvested using RIPA cell lysis buffer. The whole-cell lysate was incubated with anti-AR and anti-GFP antibodies for 12 h at 4 °C and incubated for another 4 h after addition of 30 μl protein A-agarose bead slurry (Invitrogen). After washing beads with RIPA cell lysis buffer at 4 °C, bound proteins were separated by SDS-PAGE and subjected to Western blot analysis.

GST pull-down assay

GST and GST fusion proteins were expressed in *E. coli* BL21 cells and isolated with glutathion-Sepharose-4B beads (Amersham Bioscience). Immobilized GST fusion proteins were then incubated with [^{35}S] methionine-labeled proteins produced by *in vitro* translation using the TNT-coupled transcription-translation system (Promega). Binding reactions were performed as previously described [36]. Bound proteins were eluted by addition of 20 μl of SDS-PAGE sample buffer, and then analyzed by SDS-PAGE and autoradiography [36].

Chromatin immunoprecipitation (ChIP) assay

LNCaP cells grown in RPMI media containing 5% charcoal-stripped FBS were infected with 20 MOI of recombinant adenovirus expressing SMILE and GFP, and treated with 10 nM DHT for 3 h. Cells were then cross-linked with 1% formaldehyde, and processed for ChIP assays, as previously described [36]. Anti-AR and anti-SRC-1 antibodies (Santa Cruz Biotechnology) were used for immunoprecipitation. Immunoprecipitated DNA and input-sheared DNA were subjected to PCR using a specific primer pair (forward: 5'-CATGTTCACATTAGTACACCTTGCC-3' and reverse: 5'-TCTCAGATCCAGGC TTGCTTACTGTC-3'), which amplifies a 315 bp region spanning the AR binding site of the PSA enhancer region [34]. As a negative control, PCR reactions were performed using an actin primer pair (forward: 5'-GAGACCTTCAAC ACCCCAGCC-3' and reverse: 5'-CCGTCAGGCA GCTCATAGCTC-3'), which amplifies a 362 bp region spanning exon 4 of the β-actin gene.

MTS assay

AR-positive prostate cancer cells, LNCaP and C4-2, were seeded into flat bottomed 96-well plates at a density of 2×10^3 cells per well, and cultured in RPMI media containing 5% charcoal-stripped FBS. After 48 h, cells were treated with 2, 5, and 10 mM metformin in the absence or presence of 1 nM DHT, and cultured up to 6 days. AR-negative prostate cancer PPC-1 cells were seeded into 96-well plates at a