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Leptin influences estrogen metabolism and accelerates prostate cell proliferation

Christine N. Habib [a], Ahmed M. Al-Abd [b,c], Mai F. Tolba [a,d], Amani E. Khalifa [a], Alaa Khedr [e], Hisham A. Mosli [f], Ashraf B. Abdel-Naim [a,*]

[a] Department of Pharmacology & Toxicology, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt
[b] Department of Pharmacology, Medical Division, National Research Center, Dokki, Cairo, Egypt
[c] Department of Pharmacology and Toxicology, Faculty of Pharmacy, King Abdulaziz University, Jeddah, Saudi Arabia
[d] Biology Department, School of Science and Engineering, The American University in Cairo, New Cairo, Egypt
[e] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, King Abdulaziz University, Jeddah, Saudi Arabia
[f] Department of Urology, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia

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ABSTRACT

Aim: The present study was designed to investigate the effect of leptin on estrogen metabolism in prostatic cells.
Main methods: Malignant (PC-3) and benign (BPH-1) human prostate cells were treated with 17-β-hydroxyestradiol (1 μM) alone or in combination with leptin (0.4, 4, 40 ng/ml) for 72 h. Cell proliferation assay, immunocytochemical staining of estrogen receptor (ER), liquid chromatography–tandem mass spectrometry method (LC–MS) and semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) were used.
Key findings: Cell proliferation assay demonstrated that leptin caused significant growth potentiation in both cells. Immunocytochemical staining showed that leptin significantly increased the expression of ER-α and decreased that of ER-β in PC-3 cells. LC–MS method revealed that leptin increased the concentration 4-hydroxyestrone and/or decreased that of 2-methoxyestradiol, 4-methoxyestradiol and 2-methoxyestrone. Interestingly, RT-PCR showed that leptin significantly up-regulated the expression of aromatase and cytochrome P450 1B1 (CYP1B1) enzymes; however down-regulated the expression of catechol-o-methyltransferase (COMT) enzyme.
Significance: These data indicate that leptin-induced proliferative effect in prostate cells might be partly attributed to estrogen metabolism. Thus, leptin might be a novel target for therapeutic intervention in prostatic disorders.

Introduction

Obesity has become a critical health problem worldwide [5,22]. The number of deaths attributed to obesity in the United States, is estimated to be as high as 400,000 deaths per year [20]. Several studies have shown that obesity is a risk factor for prostate cancer (PCa) and benign prostatic hyperplasia (BPH) [21,37]. Positive correlation between the body mass index and the prostate tumor volume was observed in a large sized clinical trial in Italy [4].

Several studies illustrated the relation of leptin to obesity [17,24,41]. Leptin, a 16-kDa peptide hormone and the most well characterized adipokine, is involved in reproduction, decreases caloric intake and increases energy expenditure [19,28]. Previous studies suggested the involvement of leptin in rat prostate growth [23]. It is secreted mainly from adipocytes into the blood, with a fluctuating level according to body mass. Lean individuals have an average of 4 ng/ml circulating plasma leptin, while obese ones have a higher level, around 40 ng/ml [10,32]. The expression of leptin receptors in the prostate was first demonstrated by Cioffi et al. [8]. The effect of leptin on malignant prostatic cancer cells (DU145 and PC-3) was investigated in an in-vitro study, and it illustrated that leptin causes significant growth potentiation in these cell lines [34].

Successive studies showed that not only androgens are key players in the development of prostatic cancer, but also estrogens play an important role [1,11,14,18]. The fact that the levels of androgen were found to be decreasing in the age of peak incidence of prostatic disorders while those of estrogen remained unchanged [39], led the scientists to consider estrogen as an essential co-player in prostatic disorders. Confirming this theory, was the finding that estrogen plays a significant role in breast cancer [36], which is pathophysiologically similar to prostate cancer to a great extent [30]. Prostate gland expresses two estrogen receptor subtypes (ER-α and ER-β). Activation

* Corresponding author at: Faculty of Pharmacy, Ain Shams University, Abassia, Cairo, Egypt. Tel.: +20 2 01119998505; fax: +20 2 24051107.
E-mail address: abnaim@yahoo.com, abnaim@pharma.asu.edu.eg (A.B. Abdel-Naim).

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of ER-α induces cell proliferation and inflammation, while ER-β mediates antiproliferative, anti-inflammatory and potentially anticarcinogenic effects [14]. In this context, it was reported that estrogen metabolism and the profile of estrogen metabolites have an impact on the biological effects of estrogen [15,27]. Some estrogen metabolites such as hydroxyestrogens are more potent than their parent estrogens in inducing carcinogenic effects [7]. On the other hand, methoxyestrogens possess anti-proliferative properties [31].

However, the link between leptin and estrogen metabolism remains unexplored. Therefore, the current study aimed to investigate the effect of leptin on estrogen metabolism in malignant (PC-3) and benign (BPH-1) human prostate cells.

Materials and methods

Chemicals

Human recombinant leptin was purchased from BioVision Chemical Company (Milpitas, California, USA). Sulforhodamine B (SRB), 17-β-hydroxyestradiol, dansyl chloride (Dns-Cl) 98% HPLC grade and β-glucuronidase/arylsulphatase (*Helix pomatia*, Type HP-2, ≥500 Sigma units β-glucuronidase and ≤37.5 units sulfatase activity) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). RPMI 1640 media, fetal bovine serum (FBS), antibiotics (100 μg/ml streptomycin, 100 units/ml penicillin) and phosphate-buffered saline (PBS; 0.15 M K_2HPO_4, 0.15 M Na_2HPO_4, 0.85% NaCl, pH 7.2) were purchased from Lonza Chemical Company (Walkersville, MD, USA). All other chemicals were of the highest grade commercially available.

Cell culture

Human prostate cancer cell line (PC-3) and benign prostate hyperplasic cells (BPH-1) were obtained from the National Cancer Institute (Cairo, Egypt). Cells were cultured in RPMI 1640 media and supplemented with 10% heat-inactivated FBS and antibiotics (100 μg/ml streptomycin, 100 units/ml penicillin). All cultures were maintained at 37 °C in humidified atmosphere containing CO_2 5% (v/v). Cells were harvested at 70–80% confluence and washed with PBS. Cells were detached using 0.25% trypsin in 0.1% EDTA, centrifuged at 1200 rpm for 10 min and re-suspended in growth medium in all experiments. Cell viability was checked using trypan blue exclusion assay.

Experimental design

Cells were divided into five groups for each cell line. Group I served as control group. Group II is considered as estrogen group and was exposed to 17-β-hydroxyestradiol (1 μM). Groups III, IV and V were co-treated with 17-β-hydroxyestradiol (1 μM) and 0.4, 4 and 40 ng/ml human recombinant leptin respectively.

Sulforhodamine B (SRB) proliferation assay

The SRB assay was used to assess cell proliferation as previously described [33] with minor modifications. Briefly, cells were seeded in 96-well plates at a density of 500 cells/well then treated with 17-β-hydroxyestradiol and leptin as previously described for 72 h. Media was then discarded and cells were fixed by addition of 150 μl/well cold 10% trichloroacetic acid (TCA). The plate was incubated at 4 °C for 1 h before being gently washed at least three times with tap water and then air-dried. The cells were stained by addition of 70 μl 4% SRB in 1% acetic acid for 10 min in the dark. The stain was removed and the cells were washed with 1% acetic acid, air-dried and 150 μl of 10 mM aqueous Tris base was added to dissolve the dye. The plates were shaken for 5 min until the dye was uniformly distributed and then read on an ELISA microplate reader (ChroMate™ model 4300, USA) at 545 nm. Any alteration in the number of the viable cells results in a concomitant change in the amount of dye incorporated by the cells in the culture.

Immunocytochemical (ICC) detection of estrogen receptors α & β

PC-3 and BPH-1 cells were seeded on cover slips overnight and subsequently treated with 17-β-hydroxyestradiol and leptin for 72 h as previously described. Cells attached to the cover slips were then fixed with cold ethanol 70% for 1 h. Fixed cells were washed and immersed in tris buffer saline (TBS). Permeabilization was then done by immersing slides in 3% hydrogen peroxide for 10 min. Two drops of the ready to use ER-α antibody (Novocastra Laboratories Ltd., Newcastle, UK) were applied. The concentrated ER-β (AbD Serotec, USA) was prepared according to manufacturer recommendation (1/50) and two drops were applied to each slide. Subsequently, slides were incubated in the humidity chamber overnight and poly horseradish peroxidase (HRP) enzyme conjugate was applied to each slide for 30 min. Power Stain™ 1.0 Poly HRP DAB Kit (Genemed Biotechnologies, CA, USA) was used to visualize any antigen–antibody reaction in the cells. Diaminobenzidine (DAB) chromogen was added to each slide for 2 min then rinsed, after which counterstaining with Mayer Hematoxylin was performed as the final step before slides were examined under the light microscope. The number of DAB positive cells per high power field was counted using the image analysis software (ImageJ, ver. 1.46a, NIH, USA).

Assessment of estrogen and estrogen metabolites

Cells were seeded in 6-well plates, incubated overnight and subsequently treated with 17-β-hydroxyestradiol alone or in combination with leptin (0.4, 4, 40 ng/ml) for 72 h. Estrogen and estrogen metabolites in cell media were analyzed using the liquid chromatography–tandem mass spectrometry (LC–MS) method as previously described [25]. Samples were hydrolyzed using β-glucuronidase/arylsulphatase. Dichloromethane was used to extract the steroid content then subjected to evaporation. The dried residue was dissolved in Dns-Cl and sodium bicarbonate. Samples were shaken and injected for LC–MS analysis using mixture reaction monitoring (MRM) mode. An Ion Trap 6320 MS/MS detector was used.

RNA extraction and semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA isolation was performed by using RNeasy® Mini Kit (Qiagen, Valencia, CA, USA) according to the supplier protocol. High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was undertaken for constructing a cDNA library. PCR amplification reactions were then performed using a Taq PCR Master Mix Kit (Qiagen, Valencia, CA, USA) and the following primers were used: Catechol-O-methyltransferase (COMT) sense primer, 5′ CTA-CTG-GCT-GAC-AAC-GTG-ATC-TG 3′ and the corresponding antisense primer, 5′ GTA-TTC-CAG-GAA-CGA-TTG-GTA-GTG-T 3′. Cytochrome P450 (CYP) isoform 1B1 sense primer, 5′ TTT-CGG-CTG-CCG-CTA-CA 3′ and the corresponding antisense primer 5′ ACT-CTT-CGT-TGT-GGC-TGA-GCA 3′. Aromatase sense primer, 5′ ATC-TCT-GGA-GAG-GAA-ACA-CTC-ATT-A 3′ and the corresponding antisense primer, 5′ CTG-ACA-GAG-CTT-TCA-TAA-AGA-AGG-G 3′. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as reference housekeeping gene with sense primer, 5′ CAA-GGT-CAT-CCA-TGA-CAA-CTT-TG 3′ and antisense primer 5′ GTC-CAC-CAC-CCT-GTT-GCT-GTA-G 3′. All the primers were purchased from (Metabion international AG, Martinsried, Germany). 40 cycles of PCR amplification was performed, each consisted of a denaturation step for 1 min at 94 °C, an annealing step for 30 s at 60 °C (COMT), 52 °C (CYP1B1), 58 °C (aromatase) and 58 °C (GAPDH). All PCR products were resolved by 1.5% agarose gel electrophoresis and photomicrographs were taken of the ethidium bromide-stained gels. Then gels were scanned for quantification, and the pixel intensity for each band