Leptin influences estrogen metabolism and accelerates prostate cell proliferation

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1 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 [1,14]. 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 of ER-α induces cell from adipo...
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 hydroxysterogens 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). Sulfurhodamine B (SRB), 17-β-hydroxyestradiol, dansyl chloride (Dns-Cl) 98% HPLC grade and β-glucuronidase/arylsulphatase (Heliomutus, Type HP-2, ≥ 500 Sigma units β-glucuronidase and ≤ 37.5 units sulphatase 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 KCl, 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 CO2 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 resuspended 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.

Sulfurhodamine 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-hydroxysteradiol 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 (Novoceastra 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-GCT-GTC-GAC-AAC-ATG-TG 3′ and the corresponding antisense primer, 5′-GTA-TGC-GAG-GAA-CGA-ATG-TG-3′; Cytochrome P450 (CYP) isoform 1B1 sense primer, 5′-TTT-CCG-CTT-CGG-CTA-CA 3′ and the corresponding antisense primer 5′-ACT-CTG-GAG-GAA-GAA-CTT-AT-3′; CYP1B1 sense primer, 5′-ATC-GTC-GAA-GGA-ACC-ATT-3′ and the corresponding antisense primer 5′-CTC-ACA-GAT-GTC-G-3′; Glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) was used as reference housekeeping gene with sense primer, 5′-CAA-GGT-CAT-CCA-TGA-CTA-TT-3′ and the corresponding antisense primer 5′-GTC-CAC-CCT-GCT-GTC-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 was calculated.
was determined using the image analysis software (ImageJ, ver. 1.46a, NIH, USA).

**Data analysis**

Data are presented as mean ± SEM. Multiple comparisons were performed using one-way ANOVA followed by Dunnett as a post-hoc test; the estrogen group is considered as the control group. The 0.05 level of probability was used as the criterion for significance. All statistical analyses were performed using Instat version 3 software package. Graphs were sketched using GraphPad Prism (ISI® software, USA) version 5 software.

**Results**

**Assessment of cell proliferation**

To evaluate the potential proliferative effect of leptin in malignant (PC-3) and benign (BPH-1) human prostate cells, SRB assay was used after 72 h of exposure to 17β-hydroxyestradiol (1 μM) alone or in combination with serial concentrations of leptin. It was found that leptin promoted proliferation of both cell-lines. Exposure of PC-3 cells to 0.4 ng/ml leptin didn't change cell proliferation significantly compared to the estrogen group. However, exposure to 4 and 40 ng/ml leptin significantly increased cell proliferation by 21.9% and 22.2% respectively, compared to the estrogen group (Fig. 1.A). Regarding BPH-1 cell line, exposure to 0.4 and 4 ng/ml leptin didn't induce any significant proliferative pattern, while exposure to 40 ng/ml leptin significantly (P < 0.05) induced cell proliferation by 50.9% compared to cells treated with estradiol alone (Fig. 1.B).

**Determining the expression level of estrogen receptors α & β**

To assess the effect of leptin on the expression level of ER-α & ER-β in PC-3 and BPH-1 cell lines, immunocytochemical staining was performed. In PC-3 cells, 17β-hydroxyestradiol (1 μM) alone significantly increased the number of cells expressing ER-α by 54.7%, compared to the control group (Fig. 2.A). Treatment with 0.4 & 4 ng/ml leptin didn't show significant difference in the number of cells expressing ER-α, compared to the estrogen group (Fig. 2.A). However, exposure of PC-3 cells to 40 ng/ml leptin showed significant elevation in the number of cells expressing ER-α by 72.2% compared to the estrogen group. On the other hand, leptin decreased the number of cells expressing ER-β. Exposure of PC-3 cells to 0.4 ng/ml leptin didn't show any significant difference, even though exposure to 4 & 40 ng/ml leptin significantly decreased the number of cells expressing ER-β (P < 0.05) by 28.4% and 62.5% respectively compared to the estrogen group (Fig. 2.B). The immunocytochemical staining was quantified as number of positive cells/high power field, and the figures are represented in (Fig. 2.C & D).

**Quantification of estrogen & estrogen metabolites**

To investigate the effect of leptin on estrogen metabolism within PC-3 and BPH-1 cell lines, several key metabolites (estradiol, 2- methoxyestradiol, 4-methoxyestradiol, 2-methoxyestrone, 2- hydroxyestrone, 17β-hydroxyestradiol and 4-hydroxyestrone) were measured in culture media using LC-MS. In the current work, exposure of PC-3 cells to leptin did not significantly change the levels of estradiol, 2-methoxyestrone and 4-hydroxyestrone compared to the estrogen group (Fig. 3.A). The level of 4-hydroxyestrone in the control group was under the detection limit of the method used for analysis. However, exposure to 4 and 40 ng/ml leptin significantly decreased the concentration of 2-methoxyestradiol by 37.4% and 45.3% respectively compared to the estrogen group. In addition, exposure to 0.4, 4 and 40 ng/ml leptin significantly decreased the concentration of 4-methoxyestradiol (P < 0.05) by 65.3%, 74.3% respectively compared to the estrogen group. On the other hand, 40 ng/ml leptin significantly decreased estradiol levels in BPH-1 cells by 34.9% compared to the estrogen group (P < 0.05) (Fig. 3.B). Leptin didn't significantly change the concentration of 2-methoxyestradiol compared to the estrogen group. Exposure of BPH-1 cells to 4 and 40 ng/ml leptin significantly decreased the concentration of 4-methoxyestradiol by 62% and 68.4% respectively compared to the estrogen group. Moreover, leptin decreased the concentration of 2-methoxyestrone in a dose related manner. Exposure of BPH-1 cells to 0.4, 4 and 40 ng/ml leptin decreased the concentration of 2-methoxyestrone by 96%, 99% and 99.3% respectively compared to the estrogen group. Interestingly 0.4, 4 and 40 ng/ml leptin significantly increased the concentration of 4-hydroxyestrone (P < 0.05) in BPH-1 cells by 2.3, 3.9 and 4.1 fold respectively compared to the estrogen group (Fig. 3.B). The levels of 2- hydroxyestradiol and 4-hydroxyestradiol were under the detection limit of the method used for analysis.

**Assessment of gene expression**

To explain the changes in the concentrations of some estrogen metabolites, essential enzymes involved in estrogen metabolism have been measured using RT-PCR in PC-3 and BPH-1 cell lines. Our data revealed that exposure of PC-3 cells to 0.4, 4 and 40 ng/ml leptin significantly increased aromatase expression in a non-dose related manner by 0.76, 2.6 and 1 fold compared to the estrogen group (Fig. 4.A). Furthermore, exposure to 0.4, 4 and 40 ng/ml leptin significantly up-
regulated CYP1B1 expression (P < 0.05) by 0.5, 1.3, and 1.7 fold compared to the estrogen group. On the other hand, the highest concentration of leptin (40 ng/ml) significantly down-regulated COMT expression by 48% compared to the estrogen group (Fig. 4A). Regarding BPH-1 cells, 0.4 ng/ml leptin didn't significantly change aromatase and CYP1B1 expression. Exposure to 4 and 40 ng/ml leptin significantly increased aromatase expression by 1.5 and 3.3 fold respectively, while the expression of CYP1B1 was increased by 32% and 99% respectively (P < 0.05) as compared to the estrogen group (Fig. 4B). Interestingly, estradiol (1 μM) significantly down-regulated COMT expression by 26.3% compared to the untreated cells. This down-regulation was even more profound by co-treatment with leptin, where the highest concentration of leptin (40 ng/ml) induced 60.5% suppression of COMT expression as compared to the estrogen group (Fig. 4B).

**Discussion**

Obesity is implicated in the progression of prostatic diseases such as prostate cancer (PCa) and benign prostatic hyperplasia (BPH) [13]. It is associated with greater risk for PCa-specific mortality [3,12]. Moreover, there is a growing body of evidence on the impact of estrogen and its metabolites in prostate pathophysiology. 17-β-Hydroxyestradiol is metabolized into several compounds that are different in their carcinogenic potentials. Catechol estrogens are involved in prostate cancer metabolism and progression [7], while methoxyestrogens possess anti-proliferative properties [31]. The link between leptin and estrogen metabolism has not been yet clearly demonstrated. Therefore, this study was designed to examine the effect of leptin on estrogen metabolism in malignant (PC-3) and benign human prostate cells (BPH-1).

Our results demonstrated that leptin significantly stimulated the proliferation of both PC-3 and BPH-1 cells, which are in accordance with several studies indicating that leptin had a mitogenic effect on prostate cancer cells (PC-3 and DU145) [16,34,35]. It is worth mentioning that treatment of BPH-1 cells with 17-β-hydroxyestradiol alone caused an inhibition in cell proliferation. This finding is in agreement with previous studies illustrating that estrogens had growth inhibitory effects on some cells [2,38,42].

To characterize the proliferative effects of leptin on prostate cells, PC-3 and BPH-1 cell lines were analyzed for expression of the estrogen receptor subtypes (ER-α and ER-β). ER-α is responsible for mediating the effect of estrogen in enhancing cellular proliferation. In contrast, ER-β activation results in anti-proliferative effects [1,14]. The current study illustrated that leptin significantly increased ER-α expression whereas decreased ER-β expression in PC-3 cells. This was in line with previous studies which indicated that leptin enhanced the estradiol-induced activation of ER-α in MCF-7 and HeLa cells [6] and reduced ER-β gene expression in rat prostate ventral lobe [9]. This can partly explain the proliferative effect of leptin which might be attributed to the overexpression of ER-α and down-regulation of ER-β.

Moreover, the current study revealed that leptin increased the concentration of the proliferative metabolite 4-hydroxyestrone and/or decreased that of methoxyestradiol and methoxyestron. The disturbance in balance between hydroxyestrogens and methoxyestrogens might elucidate the proliferative effect of leptin on PC-3 and BPH-1 cells. This was in accordance with our previous study demonstrating that catechol estrogens could neoplastically transform BPH-1 cells [26]. To further substantiate the changes in the concentration of estrogen metabolites, the effect of leptin on the expression of some key enzymes involved in estrogen metabolism was examined. The expression levels of aromatase, CYP1B1 and COMT enzymes were of special importance. Since, aromatase is responsible for the conversion of dihydrotestosterone (DHT) into 17-β-hydroxyestradiol while, CYP1B1 is responsible for the transformation of 17-β-hydroxyestradiol into the delirious catechol estrogen 4-hydroxyestradiol [40]. We previously reported...
that this metabolite can induce proliferation and malignant transformation of prostate cells \[26\]. COMT is of equal significance as it is the enzyme responsible for the detoxification of this noxious metabolite \[40\]. Leptin significantly increased aromatase expression. At the same time, it significantly increased the expression level of CYP1B1 which might explain the increase in the proliferative estrogen metabolite 4-hydroxyestrone in BPH-1 cells. Our results are in accordance with an earlier study that showed that leptin increases the expression level of CYP1B1 in breast cancer cell lines \[29\]. On the other hand, leptin significantly decreased the expression level of COMT which might explain the decrease in the formation of the anti-proliferative metabolites (2-methoxyestradiol, 4-methoxyestradiol and 2-methoxyestrone).

**Conclusion**

In summary, the current study demonstrated that leptin directed estrogen metabolism to generate catechol estrogens and/or decreased the formation of methoxyestrogens in malignant (PC-3) and benign (BPH-1) human prostate cells. This disturbance in balance between hydroxyestrogens and methoxyestrogens might explain the role of leptin in the progression of prostatic disorders. Further studies are needed to elucidate that leptin suppression could be a novel way to improve the efficacy of prostate disease treatments.

**Conflict of interest statement**

The authors declare that there is no conflict of interest.

**Authors’ contribution**

CN: practical part and writing the manuscript. AMA, MFT: idea of research and practical part. AK: analysis of estrogen metabolites. AEK, HAM, ABA: idea of the research and writing the manuscript.

**Uncited references**

[11,18]

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