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RESEARCH

Insulin Prevents Leptin Inhibition of RM1 Prostate Cancer Cell Growth

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Abstract The association between obesity and cancer is controversial: whereas several epidemiological, clinical and research studies using cancer cell lines have supported that high levels of insulin and leptin could favor prostate cancer development and dissemination, other studies have demonstrated opposite effects or even absence of association. The main goal of this study was to evaluate the in vitro proliferation of murine androgen insensitive prostate carcinoma cells RM1 in the presence of leptin and insulin. After assessing and confirming the presence of leptin and insulin receptors in RM1 cells by immunocytochemistry, cells were cultured in the presence of different concentrations of leptin (0, 25, 50, 100 and 200 ng/mL), insulin (0, 50, 100, 150 and 200 nM) or leptin plus insulin (25 ng/ml+50 nM; 50 ng/ml+100 nM; 100 ng/ml+150 nM; 200 ng/ml+200 nM; 25 ng/ml+150 nM; 100 ng/ml+50 nM of leptin plus insulin, respectively). Cell proliferation was evaluated by assessing the percentage of resazurin reduction, a surrogate marker of cell metabolic rate. Leptin significantly decreased the percentage of resazurin reduction in all studied concentrations while there was only a slight or non significant difference in RM1cell proliferation in the presence of insulin or insulin combined with leptin when compared with control. These results show that leptin decreases RM1 prostate cancer cell proliferation at the studied concentrations, while insulin is able to antagonize the leptin inhibition of RM1 prostate cancer cell growth in vitro. The difference in cell growth that is modulated by the various hormonal environments may explain the heterogeneous behavior of prostate cancer in the obese human population.

Keywords Prostate cancer · Obesity · Leptin · Insulin · RM1 cells · Cellular proliferation

Abbreviations

ICC	Immunocytochemistry
TNF-α	Tumor necrosis factor
IL-6	Interleucine-6
VEGF	Endothelial vascular growing factor
ObR	Leptin receptor
ObRa	Leptin receptor short isoform
ObRb	Leptin receptor long isoform
Ras/ERK1/2	Ras/extracellular signal – regulated kinases 1/2
JAK2 / STAT3	Janus kinase 2 / signal transducer and activator of transcription 3
PI-3K / Akt / GSK 3	Phosphoinositite 3 kinase / protein kinase B / glycogen synthesis kinase 3
RAS/RAF/ MEK/ERK	Ras/Raf/ mitogen-activated protein kinase/ extracellular-signal-regulated kinase pathway
Akt/PI-3K	Protein kinase B/Phosphatidylinositol 3-kinases
IGF-1	Insulin-like growth factor-1

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DIO	Diet induced obesity mice
SEM	Standard error of the mean

Introduction

Cancer is the second major cause of death worldwide, while prostate cancer is the third most common cancer in men in western countries [1].

The main risk factors that are associated with prostate cancer are age [1], family history [2], high fat diet, sedentary lifestyle and obesity [3]. Several studies concerning the last three risk factors have raised controversial results [4, 5].

Obesity is defined as an excessive accumulation of fat on adipose tissue with hazardous health implications [6] that results from genetic and environmental factors, such as diet, lifestyle and lack of physical activity [6–8]. Obesity is taking pandemic proportions and is associated with several co-morbidities, namely cardiovascular, respiratory, digestive, osteoarticular, reproductive and endocrine systems being the last two associated with the development of some cancers as, for example, prostate cancer [6, 9].

Adipose tissue is a complex energy reservoir which functions are regulated by nerves, hormones, nutrients, as well as by autocrine and paracrine mechanisms. The adipose tissue is also considered an important endocrine organ, since it produces hormones and has regulatory functions on energy balance among other endocrine and metabolic functions [10]. Adipose tissue is a dynamic organ involved in a variety of metabolic and physiologic pathways that secretes a high number of proteins named adipokines [11–13]. Some examples of adipokines include the classic cytokines related to immune system, such as, tumor necrosis factor α (TNF-α) and interleucine-6 (IL-6) [11, 12, 14]; angiotensin responsible for blood pressure regulation; adiponectin that is participates in glucose homeostasis; endothelial vascular growing factor (VEGF) that promotes angiogenesis [11–17]; and leptin which is a protein that is mainly produced and secreted by adipose tissue [18].

Leptin is secreted to plasma in proportion to the quantity of adipose tissue and the nutritional state of the individual. In general, obese individuals have high plasma leptin levels [15, 19, 20]. Leptin acts by binding to specific receptors (ObR), which have high homology to cytokine class I receptors family [21, 22]. There are six isoforms of ObR, and each isoform is expressed in different tissues in a specific way. ObRa is a short isoform that has a short intracellular domain and is expressed at a variety of cells including adipocytes and pancreatic β-cells, while ObRb is the long isoform which has a long cytoplasmatic domain which is considered the most important isoform and is highly expressed at the hypothalamus and pancreas [23]. ObRb isoform is responsible for the activation of several intracellular signalization pathways like Ras/ERK1/2 (Ras/extracellular signal-regulated kinases 1/2), JAK2/STAT3 (Janus Kinase 2/signal transducer and activator of transcription 3), and the antiapoptotic pathway PI-3K/Akt/GSK3 (phosphoinositite 3 kinase/protein kinase B/ glycogen synthesis kinase 3) [24].

Several authors defend that leptin may act as a promoter and tumor growth factor for prostate cancer cells [9, 25]. Although, several epidemiologic and clinical studies have tried to address the relationship between body adipose tissue quantity and prostate cancer, results have been controversial and largerly inconclusive [6]. This difficulty could be explained by the fact that obesity is an heterogeneous condition that is not only characterized by an excess of adipose tissue but also by the associated alterations of physiologic parameters such as insulin resistance, adipokines and androgen levels which can influence prostate cancer development [6].

Insulin is an hormone produced by pancreatic β-cells [26] that is responsible for the entrance of circulating glucose to the majority of cells in particular to the liver and muscle where glucose is stored as glycogen [27]. Several factors including obesity, sedentary lifestyle and genetic susceptibility are responsible for the development of insulin resistance which is characterized by lower glucose uptake by peripheral tissues which leads to an excess of plasma glucose that stimulates the pancreas to secrete more insulin in a way to compensate this excess and therefore being responsible for the development of hyperinsulinism [28]. Previous studies have demonstrated the presence of insulin receptors at several types of cancer cells such as prostate [29] and breast cancer [30]. It has been hypothesized that insulin could be a carcinogenic promoter by stimulating mytogenesis and inhibiting apoptosis by activation of RAS/RAF/MEK/ERK (Ras/Raf/ mitogen-activated protein kinase/ extracellular-signal-regulated kinase pathway) and Akt/PI-3K (Protein kinase B/Phosphatidylinositol 3-kinases) phosphorilation pathways after binding to the insulin receptor [31].

Hyperinsulinism is also responsible for stimulating IGF-1 (Insulin-like growth factor-1) secretion by the liver, which in vivo may act as a growth factor of multiple malign tumors, such as prostate carcinoma [31].

Increased levels of various adipokines, namely leptin, VEGF, IL-6, and TNF-α and decreased levels of adiponectin, have been associated with several different carcinogenic mechanisms including cellular differentiation, apoptosis, cellular proliferation, angiogenesis and alterations of sex steroids levels. Numerous studies support that leptin promotes prostate cancer development while adiponectin could instead have a protective role [32]. High levels of

inflammatory cytokines, such as Il-6 and TNF-α, were also considered potential risk factors to the development of prostate cancer. In another way, TNF-α induce IL-6 and leptin release interfering also with insulin signaling pathway by the activation of resistance to the effects of this hormone [33].

In a previous in vivo study of ours [34], in which obese mice, namely *ob/ob* mice, *db/db* mice and mice with diet induced obesity (DIO), and normoponderal C57BL/6J mice used as controls, were inoculated subcutaneously with RM1 androgen insensitive murine prostate cancer cells, we have demonstrated that leptin deficient *ob/ob* and DIO mice developed larger tumors, while hyperleptinemic and insulin deficient *db/db* mice developed smaller tumors when compared with controls. Additionally, there was a negative correlation between leptin plasma levels and tumor size and the cell proliferation indexes suggesting that leptin does not act as tumor proliferative promoter but as an inhibitor of RM1 cell proliferation [34]. Furthermore, we have demonstrated that hyperinsulinemic and leptin deficient *ob/ob* mice were the group that presented larger tumors when compared with controls suggesting that insulin could have a role in promoting RM1 cell proliferation or antagonized the anti-proliferative effects of leptin [34].

In sequence of the aforementioned study, the main goal of our current study was to assess the effects of leptin and insulin on RM1 cell proliferation in vitro, without the presence of other confounding circulating factors that are present in vivo.

Material and Methods

Cell Culture

Murine androgen insensitive RM1 prostate carcinoma cells that were obtained from tumors induced in mice were kindly offered by Prof T. Thompson from MD Anderson Cancer Center, Houston, Texas, USA [35].

Cells were grown at 25 cm^2 flasks containing Dulbecco's Modified Essential Medium (DMEM—Invitrogen, New York, NY, USA) to which was added sodium bicarbonate (Sigma, St Louis, MO, USA), Penicillin-Streptomycin 100 U/mL (Invitrogen, Auckland, N.Z.), Nystatine 100 U/mL (Sigma, Auckland, N.Z.) and 50 mM HEPES buffer solution (Invitrogen, Auckland, N.Z.). The cells in culture medium were placed in an incubator (Function Line—Heraeus Instruments, Hanau, Germany) which maintained a constant temperature 37°C and humid atmosphere with 5% CO_2. Every time cells became confluent they were passaged using trypsin 0,25% (Invitrogen, Auckland, N.Z.). Cellular viability was evaluated using the trypan blue (Sigma, USA) test and a viability superior to 90% was considered satisfactory.

RM1 Cell Characterization

Immunocytochemistry for Leptin Receptor

To verify the presence of leptin receptor (ObR) at RM1 cells membrane an immunocytochemical study was done. A cytospin (Cytospin 3—Shandon, Cheshire, UK) of 100 μL RM1 cells suspension at 12th passage was performed on poly-L-lysine (Sigma) microscope slides and the cell preparation was fixed with pure acetone. Prostate of normal C57BL/6J mice was collected and processed for routine histological analysis after being fixed in a paraformaldehyde and picric acid 14% solution, to be used as control. Antigen retrieval was performed using microwaves with incubation of microscope slides at citrate buffer solution 0,1 M at pH 6.

After washing the prostate and RM1 cells slides with PBS, endogenous peroxidases were blocked by emerging slides at hydrogen peroxide solution 0.3% for 10 min. Unspecific bindings were blocked with PBS 1% BSA solution during 30 min, then slides were incubated, overnight, with the primary antibody anti-ObR (ObR H-300: sc-8,325—Santa Cruz Biotechnology, CA, USA) (1:100) at 4°C followed by the secondary antibody (Polyclonal swine anti-rabbit, DaKoCytomation, Glostrup, Denmark) (1:100) incubation for 30 min at room temperature. After washing with PBS the slides were incubated with ABC (DaKoCytomation) complex during 30 min, they were then washed again in PBS and were revealed with DAB (DaKoCytomation). Internal negative control was obtained by omission of the primary antibody.

Immunocytochemistry for Insulin Receptor

The presence of insulin receptor at RM1 cells was evaluated by using Novolink Min Polymer Detection System (Newcastle, UK) for immunocytochemistry.

Slides were prepared as described above for leptin receptor immunocytochemistry until inhibition of endogenous peroxidase. After washing slides with PBS unspecific bindings were blocked with Protein Block (Novolink Polymer Detection System, Newcastle, UK). The slides were incubated with primary antibody (Insulin Receptor R antibody [EP327Y] (ab40782—Abcam, Cambridge, UK) overnight at 4°C. After washing with PBS, slides were incubated during 30 min with Post Primary Block (Novolink Polymer Detection System, Newcastle, UK) followed by slides revelation with DAB (DaKoCytomation).

Internal negative control was obtained by omission of the primary antibody, absence of staining in mice cerebrum was used a negative control while prostate and kidney of C57BL/6J mice were used as positive controls.

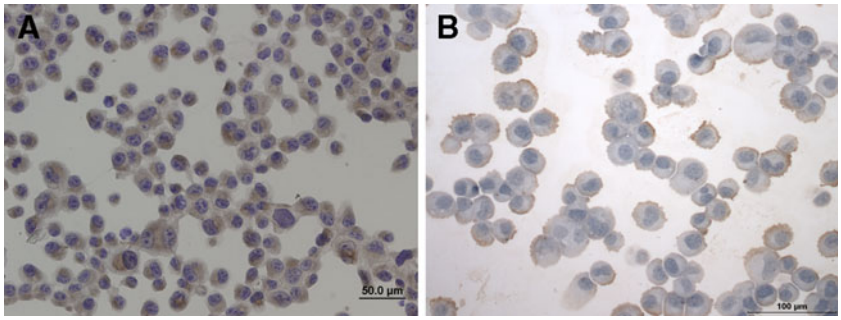

Fig. 1 Immunocytochemistry of RM1 cells for OBRb using the specific antibody OBR 12-A, **a** and for insulin receptor using anti-insulin receptor antibody **b** depicts a brown coloration of the RM1 cells on the cell periphery which supports the OBRb and insulin receptor presence at this prostate cancer cellular line (200×)

In Vitro Evaluation of Cell Proliferation

In vitro cellular proliferation was evaluated by an indirect method of cell growth, the resazurin reduction method that detects the reduction of the intervenient molecules in the electron transport chain ($FMNH_2$, $FADH_2$, NADH, NADPH and cytochromes), since rezasurine has the highest redox potential of all.

RM1 Cell Growth Dose–Response Curve

To evaluate the ideal cell concentration to be used in the following studies a dose–response curve was performed. Three different cell concentrations (2×10^4, 4×10^4 and 7×10^4 cells/mL) were seeded at wells of a plaque (24 well cell culture cluster—Costar, NY, USA) containing culture medium, five duplicates (5 wells) for each concentration were used. Negative control was done by preparing five wells without cells, containing cell culture medium only. At each well (20 in total) was added 200 μL of resazurin (AbD Serotec, Oxford, UK). At each time point 200 μL of medium was collected from each well and was transferred to an ELISA plaque, absorbance was measured at 570 and 600 nm. The absorbance was measured at time 0, 2, 4, 6, 12 and 22 h after.

The % of rezasurine reduction was calculated by using the following equation:

$$\%\text{reduction} = (\text{Eox}_{600\text{nm}} \text{ x } \text{abs}_{570\text{nm}} - \text{Eox}_{570\text{nm}} \text{ x } \text{abs}_{600\text{nm}} / \text{Ered}_{570\text{nm}} \text{ x } \text{abs}_{\text{branco } 600\text{nm}} - \text{Ered}_{600\text{nm}} \text{ x } \text{abs}_{\text{branco } 570\text{nm}}) \text{ x } 100, \text{being } \text{Eox}_{570\text{nm}} = 80,573, \text{Eox}_{600\text{nm}} = 117,216, \text{Ered}_{570\text{nm}} = 155,667 \text{ and } \text{Ered}_{600\text{nm}} = 14,652$$

RM1 Cell Growth in Presence of Leptin, Insulin or Both Leptin and Insulin

After choosing the ideal cell concentration (2×10^4 cells/mL) new plaques were prepared as mentioned before. Cells were cultured with five different leptin (Bachem, Germany) concentrations that were added the culture medium (0, 25, 50, 100 and 200 ng/mL) together with 200 μL of resazurin (AbD Serotec, Oxford, UK), or human insulin (Insuman rapid—Sanofi Aventis) in five different concentrations (0, 50, 100, 150 e 200 nM), or leptin plus insulin in different concentrations, namely 25 ng/mL leptin plus 50 nM insulin, 50 ng/mL leptin plus 100 nM insulin, 100 ng/mL leptin plus 150 nM insulin, 200 ng/mL Leptin plus 200 nM insulin, 25 ng/mL leptin plus 150 nM insulin and 100 ng/mL leptin plus 50 nM insulin. Five duplicates were prepared for each concentration and at each well was added 200 μL of resazurin (AbD Serotec, Oxford, UK). At each time point, namely time 0, 2, 4, 6, 12 and 22 h after, 200 μL of medium was collected from each well and was transferred to an ELISA plaque, the absorbance was measured at 570 and 600 nm and the % of resazurin reduction was calculated by using the same equation as previously described.

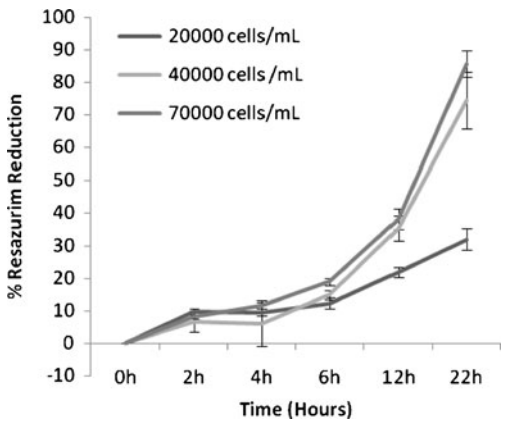

Fig. 2 Dose response curve of cell proliferation evaluated by the determination of the % resazurin reduction (change from baseline) at different time points during 22 h of three different initial cell concentrations (2000, 40 000 and 70 000 cells/mL)

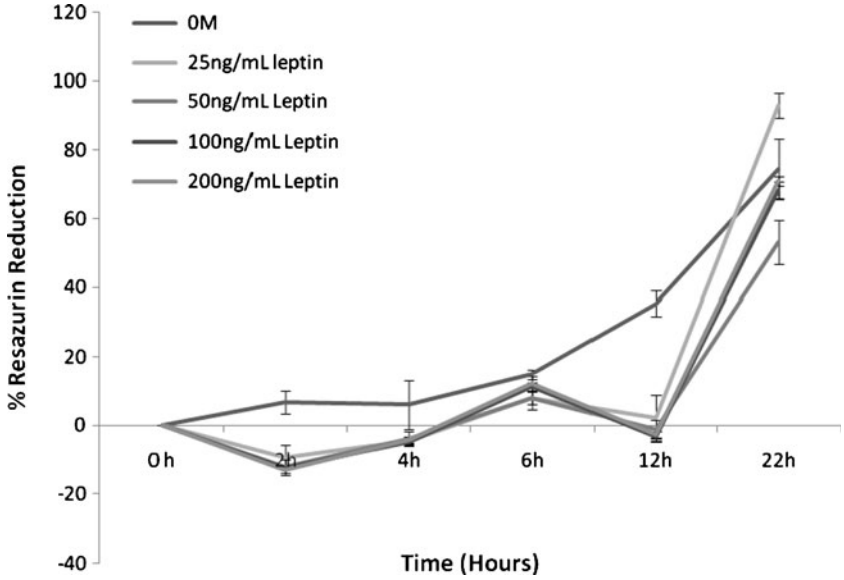

Fig. 3 Graphic representing the change from baseline of the % of resazurin reduction in the culture medium of RM1 cells in the presence of the different leptin concentrations, with the values of the mean±standard error of the mean at the different time points for the different concentrations of leptin used (25, 50, 100 and 200 ng/mL)

Statistical Analysis

All statistically analyses were done using SPSS software (version 17) for Windows. Means, standard error of the mean (SEM), normality and homogeneity of variances were calculated for each group. ANOVA test with post hoc Bonferroni correction when appropriate was used to compare the differences between means. $P<0.05$ was considered statistical significant.

Results

RM1 Cell Characterization

Immunocytochemistry for Leptin and Insulin Receptor

The presence of leptin receptors was demonstrated by immunocytochemistry using an antibody anti-ObRa and ObRb. RM1 cells displayed a brown coloration at the cells periphery, which corresponds to the leptin receptors location (Fig. 1A). RM1 cells also presented a positive stain after the insulin receptor immunocytochemistry observed by the appearance of brown coloration at cell membranes where insulin receptors are located (Fig. 1B).

Cellular Proliferation

RM1 Cell Growth Dose–Response Curve

A dose–response study was performed to choose the ideal cell concentration to study cell growth when cultured with leptin or insulin, by assessing the % of resazurin reduction as an indirect method to assess cell proliferation. From the analysis of the dose–response curve (Fig. 2), the cell concentration chosen was of 2×10^4 cells/mL since with this cell concentration there was a linear increase in the % of resazurin reduction that was not observed with the lower cell concentration, while there was no significant increase in the % of rezasurine reduction by increasing the cell concentration.

Table 1 RM1 cell growth, in the presence of different leptin concentrations (0,25,50, 100 and 200 ng/mL) added to the cell culture medium, displayed significant decrease in the % of rezasurine reduction from the first evaluation at 2h and onwards for all studied leptin concentrations when compared with the control group

[leptin] (ng/mL)	Time 0 0 h	Time 1 2 h	Time 2 4 h	Time 3 6 h	Time 4 12 h	Time 5 22 h
0	0	6.74±3.27	6.10±7.13	14.93±1.35	35.45±3.77	74.48±8.81
25	0	−9.33±3.54***	−4.60±1.23***	7.99±3.20*	2.17±6.78***	93.03±3.76**
50	0	−12.22±2.37***	−3.91±2.06**	8.70±1.92 *	−1.16±2.70***	53.20±6.41***
100	0	−12.41±0.47***	−4.70±0.57***	11.23±1.38	−3.28±1.59***	68.37±2.42
200	0	−13.09±0.80***	−4.27±0.74***	12.40±2.14	−2.71±0.98***	71.05±1.43

(*$p<0.05$; **$p<0.01$; ***$p<0.001$)

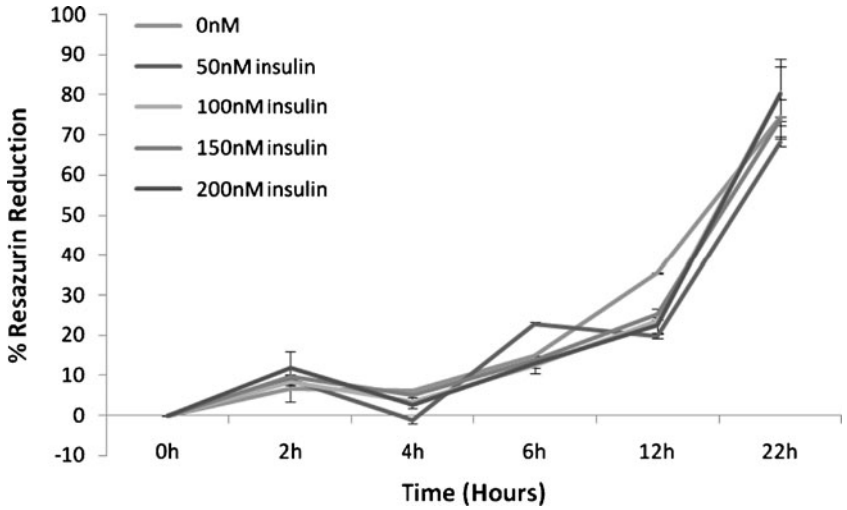

Fig. 4 Graphic representing the change from baseline of the % of resazurin reduction in the culture medium of RM1 cells in the presence of the different insulin concentrations, with the values of the mean±standard error of the mean at the different time points for the different concentrations of insulin used (50, 100, 150 and 200 nM)

RM1 Cell Growth in Presence of Leptin and Insulin

RM1 cell growth, in the presence of different leptin concentrations (0, 25, 50, 100 and 200 ng/mL) added to the cell culture medium, displayed significant decrease in the % of rezasurine reduction from the first evaluation at 2 h and onwards for all studied leptin concentrations when compared with the control group ($p<0{,}001$) (Fig. 3, Table 1). RM1 cell culture in the presence of different insulin concentrations (0, 50, 100, 150 and 200 nM) added to the medium, has also yielded a significant decrease in the % of resazurin reduction although smaller and only after 12 h of incubation ($p<0.01$), except for the lower insulin concentration (50 nM) that decreased cell proliferation from the first time point (2 h) onwards ($p<0.05$) (Fig. 4, Table 2). RM1 cell growth in presence of leptin plus insulin in the different studied concentrations (25 ng/mL leptin plus 50 nM insulin, 50 ng/mL leptin plus 100 nM insulin, 100 ng/mL leptin plus 150 nM insulin, 200 ng/mL leptin plus 200 nM insulin, 25 ng/mL leptin plus 150 nM insulin, 100 ng/mL leptin plus 50 nM insulin) overall was not significantly different from control (Fig. 5. Table 3).

Discussion

The association between obesity and prostate cancer is considered controversial, since many studies that have tried to correlate circulatory factors that are altered in obesity with prostate cancer, have yielded contradictory results [9, 36, 37]. The main goal of this study was to evaluate the effect of leptin and insulin on androgen insensitive prostate cancer RM1 cells growth in vitro.

The RM1 cells where characterized regarding to the presence of leptin receptors by immunocitochemistry using a specific primary antibody anti-ObR short and long receptor isoforms, ObRa and ObRb respectively. After the immunocitochemistry, RM1 cells displayed a brown staining at the cell periphery corresponding to the location of the leptin receptors at the cell membrane, similar to what was observed in the prostate epithelial glandular cells of C57Bl/6 mice known to express leptin receptors as previously demonstrated by other authors and used as a positive control [38]. In the present study, in which the murine androgren insensitive prostate carcinoma cells RM1 were cultivated in the presence of

Table 2 RM1 cell culture in the presence of different insulin concentrations (0, 50, 100, 150 and 200 nM) added to the medium, has yielded a significant decrease in the % of rezasurin reduction only after 12h of incubation, except for the lower insulin concentration (50 nM) that decreased cell proliferation from the first time point (2h) onwards

[insulin] (nM)	Time 0 0 h	Time 1 2 h	Time 2 4 h	Time 3 6 h	Time 4 12 h	Time 5 22 h
0	0	6.74±3.27	6.10±7.13	14.93±1.35	35.45±3.77	74.48±8.81
50	0	8.81±0.41	−1.32±0.67*	22.84±0.39*	19.71±0.53***	68.36±1.33
100	0	8.24±0.76	3.47±0.96	12.50±2.02	23.57±3.02**	80.62±8.22
150	0	9.63±0.74	5.05±0.61	13.85±1.00	25.29±1.42**	73.98±4.82
200	0	11.92±4.06	2.55±0.75	12.84±1.06	22.36±1.99***	80.25±6.78

(*$p<0.05$; **$p<0.01$; ***$p<0.001$)

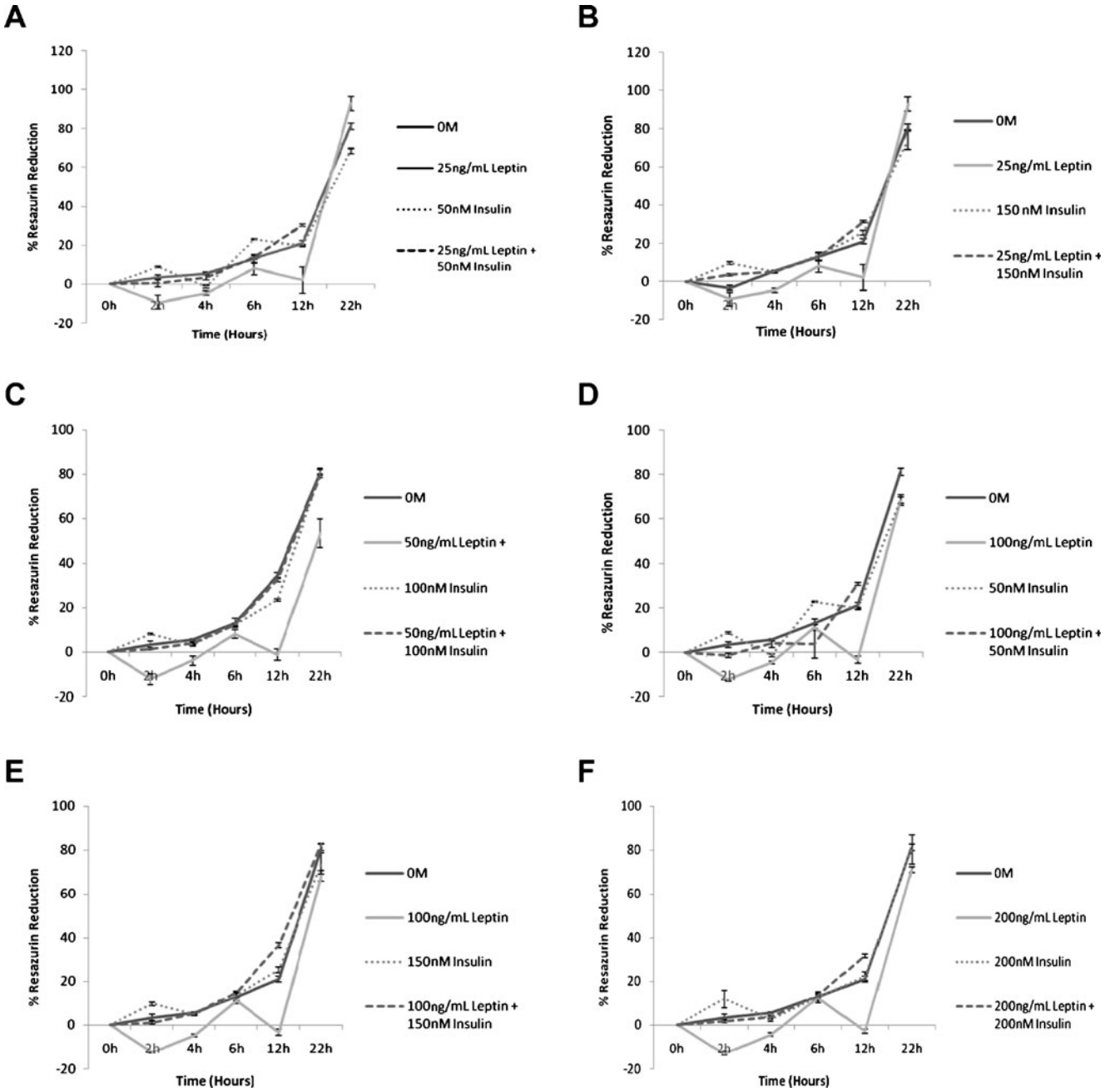

Fig. 5 Graphic representing the change from baseline of the % of resazurin reduction in the culture medium of RM1 cells in the presence of the different concentrations of leptin plus insulin, with the values of the mean±standard error of the mean at the different time points for the different concentrations of leptin plus insulin used: 25 ng/ml leptin+50 nM insulin **a**, 25 ng/mL leptin+150 nM insulin **b**, 50 ng/mL leptin+100 nM insulin **c**, 100 ng/mL leptin+50 nM insulin **d**, 100 ng/mL leptin+150 nM insulin **e**, 200 ng/mL leptin+200 nM insulin **f**

different leptin concentrations added to the cell culture medium, we were able to demonstrate that leptin inhibits cell proliferation, since % of rezasurine reduction was significantly reduced in the presence of leptin when compared to control. This observation contrasts with previous studies where leptin has been shown to induce in vitro proliferation of androgen independent human prostate carcinoma cell lines DU-145 and PC-3 [39], whereas Deo et al., using different prostate cancer cell lines cultivated in the presence of leptin, namely androgen independent DU-145 and PC-3 cells, and androgen dependent cell LNCaP, were able to demonstrate that leptin increases cell proliferation and activates intracellular pathways signals only in the androgen dependent cell line LNCaP [40].

Table 3 RM1 cell growth in presence of leptin plus insulin in the different studied concentrations was not significantly different from control

[Leptin] (ng/mL)	[Insulin] (nM)	Time 0 0 h	Time 1 2 h	Time 2 4 h	Time 3 6 h	Time 4 12 h	Time 5 22 h
0	0	0	3.45±1.40	5.55±0.42	13.04±2.10	34.58±1.31	81.17±1.57
25	50	0	0.52±1.83	3.33±1.30	13.70±0.95	30.45±0.65	74.55±0.68
25	150	0	3.53±0.52	5.29±0.43	13.19±0,26	31.22±0.58	71.22±1.43
50	100	0	1.55±0.52	4.11±0.65	12.35±0.73**	32.63±0.97	79.27±0.83
100	50	0	−1.35±0.89	4.08±1.90	3.89±6.50	30.72±0.70	72.18±0.75
100	150	0	1.06±0.54	5.17±0.25	15.08±0.59	36.62±1.29	82.94±0.41
200	200	0	1.63±0.63	3.52±0.70	13.63±1.07	31.59±0.88	76.50±0.88

(**$p<0.01$)

Epidemiological studies in which circulatory factors known to be altered by the obese state were measured in patients with prostate cancer and healthy individuals have not found any significant association between the leptin levels and the risk for the development of prostate cancer, however a positive correlation was found between insulin plasma levels and the risk for prostate cancer [41].

Therefore, in the present study, after demonstrating the insulin receptor presence in RM1 cells by ICC using a specific anti-insulin receptor primary antibody, we have also evaluated the insulin effect on RM1 cell growth in vitro. We have found that insulin, although in a less pronounced way, is also able to decrease significantly RM1 cell proliferation, in contrast to the results obtained by Hsing et al. that found that insulin but not leptin levels were associated with prostate cancer risk in man [41].

Furthermore, in order to test whether there was any synergism between leptin and insulin, RM1 cells were cultured simultaneously in the presence of different concentrations of leptin plus insulin, the percentage of rezasurin reduction in the RM1 cell culture medium was no different from control.

The results obtained in the herein study corroborate the results obtained in our previous in vivo study [34]. In that previous work, we have inoculated subcutaneously with RM1 different obese mice models, namely *ob/ob* mice, *db/db* mice and mice with diet induced obesity (DIO), while normoponderal C57BL/6J mice were used as controls. We demonstrated then that leptin deficient *ob/ob* and DIO mice developed larger tumors while hyperleptinemic and insulin deficient *db/db* mice developed smaller tumors when compared with controls. Additionally, there was a negative correlation between leptin plasma levels and tumor size and the cell proliferation indexes evaluated suggesting that leptin does not act as tumor proliferative promoter but rather as an inhibitor of RM1 cell proliferation [34]. Furthermore, we have shown that hyperinsulinemic and leptin deficient *ob/ob* mice were the group that presented larger tumors when compared with controls suggesting that insulin could have a role in promoting RM1 cell proliferation or prevented the anti-proliferative effects of leptin [34].

In our current study we were able to demonstrate that leptin depict an anti-proliferative effect on RM1 cell growth, as well as insulin, although in a less pronounced way, and also that insulin hindered the anti-proliferative effects of leptin in tumor cell growth, as it was suggested by the in vivo study where *ob/ob* mice presenting high levels of insulin and leptin deficiency presented significantly larger tumors when compared with tumors of control mice (C57BL/6J) [34].

Although obesity is a medical condition with rising prevalence, it is far from representing a homogeneous population characterized by an increased body mass index [42]. With the rare exception of individuals with congenital leptin deficiency, obesity is associated with increased levels of leptin that are proportional to the fat mass, whilst insulin levels may vary depending on the degree of insulin resistance which may not be linearly related to the obese state [43]. Even though most obese individuals have high levels of leptin and insulin, depending on genetic factors, there are some lean individuals with high insulin levels and obese individuals with low insulin levels, which may act as a confounding factor in human based epidemiologic studies [44]. Additionally, the insulin resistant state over the years may be associated with pancreatic β cell failure and insulin deficiency that usually characterizes the development of diabetes mellitus [45]. Remarkably, diabetes mellitus has been associated with a lower risk factor for prostate cancer [46, 47].

In conclusion, our in vitro study suggests that leptin inhibits cell proliferation and insulin seams to antagonize the anti-proliferative effects of leptin, which may explain the heterogeneity of results arising from the human studies.

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References

1. Kumar RJ, Barqawi AB, Craw ED (2008) Epidemiology of Prostate Cancer. US Oncology Review 2005
2. Schaid DJ (2004) The complex genetic epidemiology of prostate cancer. Hum Mol Genet 13(1):103–121
3. Mukherjee P, Sotnikov AV, Mangian HJ et al (1999) Energy intake and prostate tumor growth, angiogenesis, and vascular endothelial growth factor expression. J Natl Cancer Inst 91 (6):512–523
4. Bosland MC, Oakley-Girvan I, Whittemore AS (1999) Dietary fat, calories, and prostate cancer risk. J Natl Cancer Inst 91(6):489–491
5. Friedenreich CM, Orenstein MR (2002) Physical activity and cancer prevention: etiologic evidence and biological mechanisms. J Nutr 132(11 Suppl):3456S–3464S
6. Calle EE, Kaaks R (2004) Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. Nat Rev Cancer 4(8):579–591
7. Crespo CJ, Smit E, Troiano RP et al (2001) Television watching, energy intake, and obesity in US children: results from the third National Health and Nutrition Examination Survey, 1988–1994. Arch Pediatr Adolesc Med 155(3):360–365
8. Stubbs CO, Lee AJ (2004) The obesity epidemic: both energy intake and physical activity contribute. Med J Aust 181(9):489–491
9. Mistry T, Digby JE, Desai KM et al (2007) Obesity and prostate cancer: a role for adipokines. Eur Urol 52(1):46–53
10. Garg A (2006) Adipose tissue dysfunction in obesity and lipodystrophy. Clin Cornerstone 8(Suppl 4):S7–S13
11. Trayhurn P, Beattie JH (2001) Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ. Proc Nutr Soc 60(3):329–339
12. Kershaw EE, Flier JS (2004) Adipose tissue as an endocrine organ. J Clin Endocrinol Metab 89(6):2548–2556
13. Ronti T, Lupattelli G, Mannarino E (2006) The endocrine function of adipose tissue: an update. Clin Endocrinol (Oxf) 64(4):355–365
14. Wajchenberg BL (2000) Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. Endocr Rev 21(6):697–738
15. Haque WA, Garg A (2004) Adipocyte biology and adipocytokines. Clin Lab Med 24(1):217–234
16. Tilg H, Moschen AR (2006) Adipocytokines: mediators linking adipose tissue, inflammation and immunity. Nat Rev Immunol 6 (10):772–783
17. Badman MK, Flier JS (2007) The adipocyte as an active participant in energy balance and metabolism. Gastroenterology 132(6):2103–2115
18. Zhang Y, Proenca R, Maffei M et al (1994) Positional cloning of the mouse obese gene and its human homologue. Nature 372 (6505):425–432
19. Frederich RC, Hamann A, Anderson S et al (1995) Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action. Nat Med 1(12):1311–1314
20. Boden G, Chen X, Mozzoli M et al (1996) Effect of fasting on serum leptin in normal human subjects. J Clin Endocrinol Metab 81(9):3419–3423
21. Bjorbaek C, Uotani S, da Silva B et al (1997) Divergent signaling capacities of the long and short isoforms of the leptin receptor. J Biol Chem 272(51):32686–32695
22. Tartaglia LA (1997) The leptin receptor. J Biol Chem 272 (10):6093–6096
23. Hoggard N, Hunter L, Duncan JS et al (1997) Leptin and leptin receptor mRNA and protein expression in the murine fetus and placenta. Proc Natl Acad Sci U S A 94(20):11073–11078
24. Bailleul B, Akerblom I, Strosberg AD (1997) The leptin receptor promoter controls expression of a second distinct protein. Nucleic Acids Res 25(14):2752–2758
25. Somasundar P, Frankenberry KA, Skinner H et al (2004) Prostate cancer cell proliferation is influenced by leptin. J Surg Res 118 (1):71–82
26. Banting FG (1926) An Address on Diabetes and Insulin: Being The Nobel Lecture Delivered at Stockholm on September 15th, 1925. Can Med Assoc J 16(3):221–232
27. Karam JH, Grodsky GM, Forsham PH (1968) Insulin secretion in obesity: pseudodiabetes? Am J Clin Nutr 21(12):1445–1454
28. Kahn BB, Flier JS (2000) Obesity and insulin resistance. J Clin Invest 106(4):473–481
29. Cox ME, Gleave ME, Zakikhani M et al (2009) Insulin receptor expression by human prostate cancers. Prostate 69(1):33–40
30. Cullen KJ, Yee D, Sly WS et al (1990) Insulin-like growth factor receptor expression and function in human breast cancer. Cancer Res 50(1):48–53
31. Frasca F, Pandini G, Sciacca L et al (2008) The role of insulin receptors and IGF-I receptors in cancer and other diseases. Arch Physiol Biochem 114(1):23–37
32. Baillargeon J, Rose DP (2006) Obesity, adipokines, and prostate cancer (review). Int J Oncol 28(3):737–745
33. Danforth KN, Rodriguez C, Hayes RB et al (2008) TNF polymorphisms and prostate cancer risk. Prostate 68(4):400–407
34. Ribeiro AM, Andrade S, Pinho F et al (2010) Prostate cancer cell proliferation and angiogenesis in different obese mice models. Int J Exp Pathol 91(4):374–386
35. Baley PA, Yoshida K, Qian W et al (1995) Progression to androgen insensitivity in a novel in vitro mouse model for prostate cancer. J Steroid Biochem Mol Biol 52(5):403–413
36. Freedland SJ, Giovannucci E, Platz EA (2006) Are findings from studies of obesity and prostate cancer really in conflict? Cancer Causes Control 17(1):5–9
37. Giovannucci E, Rimm EB, Liu Y et al (2003) Body mass index and risk of prostate cancer in U.S. health professionals. J Natl Cancer Inst 95(16):1240–1244
38. Malendowicz W, Rucinski M, Macchi C et al (2006) Leptin and leptin receptors in the prostate and seminal vesicles of the adult rat. Int J Mol Med 18(4):615–618
39. Hoda MR, Popken G (2008) Mitogenic and anti-apoptotic actions of adipocyte-derived hormone leptin in prostate cancer cells. BJU Int 102(3):383–388
40. Deo DD, Rao AP, Bose SS et al (2008) Differential effects of leptin on the invasive potential of androgen-dependent and -independent prostate carcinoma cells. J Biomed Biotechnol 2008:163902
41. Hsing AW, Chua S Jr, Gao YT et al (2001) Prostate cancer risk and serum levels of insulin and leptin: a population-based study. J Natl Cancer Inst 93(10):783–789
42. Kelly T, Yang W, Chen CS et al (2008) Global burden of obesity in 2005 and projections to 2030. Int J Obes (Lond) 32(9):1431–1437
43. Farooqi IS, O'Rahilly S (2009) Leptin: a pivotal regulator of human energy homeostasis. Am J Clin Nutr 89(3):980S–984S
44. Belkina AC, Denis GV (2010) Obesity genes and insulin resistance. Curr Opin Endocrinol Diabetes Obes 17(5):472–477
45. Muoio DM, Newgard CB (2008) Mechanisms of disease: molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes. Nat Rev Mol Cell Biol 9 (3):193–205
46. Wotton CJ, Yeates DG, Goldacre MJ (2011) Cancer in patients admitted to hospital with diabetes mellitus aged 30 years and over: record linkage studies. Diabetologia 54(3):527–534
47. Polednak AP, Phillips CE (2010) Obtaining data on comorbid diabetes among patients in a U.S. population-based tumor registry. J Registry Manag 37(2):57–64