Original Paper

Chronic-Leptin Attenuates Cisplatin Cytotoxicity in MCF-7 Breast Cancer Cell Line

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Key Words

Oxidative stress • Leptin • Breast cancer • Cell growth • Sirtuin1 • Cisplatin drug

Abstract

Background/Aims: Large-scale epidemiological studies support a correlation between obesity and breast cancer in postmenopausal women. Circulating leptin levels are increased in obese and it has been suggested to play a significant role in mammary tumor formation and progression. Moreover, regulation of oxidative stress is another important factor in both tumor development and responses to anticancer therapies. The aim of this study was to examine the relationship between oxidative stress and chronic leptin exposure. **Methods:** We treated MCF-7 breast cancer cells with 100 ng/mL leptin for 10 days and analyzed cell growth, ROS production and oxidative damage, as well as, some of the main antioxidant systems. Furthermore, since the hyperleptinemia has been associated with a worse pathology prognosis, we decided to test the influence of leptin in response to cisplatin anticancer treatment. **Results:** Leptin signalling increased cell proliferation but reduced ROS production, as well as, oxidative damage. We observed an upregulation of SIRT1 after leptin exposure, a key regulator of stress response and metabolism. Additionally, leptin counteracted cisplatin-induced cytotoxicity in tumor cells, showing a decrease in cell death. **Conclusion:** Chronic leptin could contribute to the effective regulation of endogenous and treatment-induced oxidative stress, and it contributes to explain in part its proliferative effects.

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Introduction

Adipose tissue-derived signalling molecules, including adipokines, are emerging as key candidate molecules that link obesity with cancer [1, 2]. Obesity commonly leads to adipokine deregulation, and an appreciable number of cancer-related studies have focused on the impact of adipokines on cancer progression and metastasis [3].

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Leptin, discovered and characterized by Friedman et al. in 1994, is encoded by the human obese (ob) gene [4], and is one of the most important adipokines synthesized mainly by the white adipose tissue, although other cell types such as gastric mucosa, skeletal muscle, mammary epithelium, placenta, bone marrow and pituitary, secrete low quantities [5]. This hormone is considered to be a key marker of nutritional status and energy metabolism, acting as a regulator of food intake and energy expenditure via hypothalamic-mediated effects [6]. Leptin serum levels increase with the amount of body fat, and this hormone has been reported to induce pro-inflammatory responses [7]. Leptin acts mainly by the leptin receptor (Ob-R), which has six isoforms, and is a product of the diabetes (db) gene [4, 8]. The longest form of the OB-Rb receptor has full signalling capabilities and is able to activate the Jak/STAT pathway, the main one used by leptin to exert its effects [9].

Although both leptin and its Ob-R are necessary for mammary gland development, they are expressed in reduced amounts by the epithelial cells of normal human mammary glands [10, 11]. In contrast, a number of studies have reported that both leptin and Ob-R are over-expressed in breast cancer, and are related to poor prognosis in patients with this malignancy [9, 12]. Specific leptin-induced signalling pathways are involved in the increase in levels of the inflammatory, mitogenic, anti-apoptotic and pro-angiogenic factors in breast cancer [7]. In this context, evidence that obese women have a higher risk for breast cancer and that obesity is in turn characterized by high levels circulating leptin seems to indicate a correlation between leptin and breast cancer risk [13, 14].

ROS generation and depletion of antioxidant systems cause oxidative stress [15]. Cancer cells are under high levels of oxidative stress due to alterations in several signalling pathways that affect cellular metabolism [16, 17]. However, through additional mutations and adaptations, cancer cell exerts tight regulation of ROS and antioxidants to ensure cell survival, and ROS levels are reduced to moderate levels [18, 19]. Therefore, the effective regulation of endogenous and treatment-induced oxidative stress is an important factor in both tumor development and the responses to anticancer therapies [16], such as cisplatin (cis-diamminedichloroplatinum II or CDDP) [20, 21]. CDDP is a widely used antineoplastic agent, which produces adducts between adjacent purines in DNA, leading to replication arrest, transcription inhibition, cell-cycle arrest, DNA repair and apoptosis [22]. Nevertheless, recent evidences have reported that DNA damage is minimally involved in the cell death program induced by CDDP [23, 24]. It seems that the oxidative damage on cell membrane proteins and thiol-containing molecules, and the changes of energy metabolism contribute significantly to CDDP cytotoxicity via mitochondrial cell death pathways.

Nowadays, around 60% of breast carcinomas are hormone-dependent [25, 26]. Thus, the use of antiestrogens, such as tamoxifen, as therapy for estrogen-responsive breast cancers has been used for the last three decades. However, great majority of patients with advanced disease eventually develop resistance to antiestrogen drugs [27]. Although clinical trials of the effect of CDDP in antiestrogen-resistant patients have not yet been conducted, studies in cancer cell lines are showing promising results. These studies have reported the potential of CDDP to provide a novel chemotherapy strategy in antiestrogen-resistant breast cancer patients [28, 29].

On the other hand, cancer cells must rewire cellular metabolism to satisfy the energetic demand that is required to support rapid growth and proliferation. There is some debate about the selective advantage that glycolytic metabolism provides to proliferating tumor cells [30]. Initial work focused on the concept that tumor cells develop defects in mitochondrial function, and that aerobic glycolysis is therefore a necessary adaptation to cope with a lack of ATP generation by oxidative [31]. However, it was found that several tumors retain capacity for oxidative phosphorylation [30]. Furthermore, mitochondrial function in cell metabolism is not just restricted to ATP production for cellular demands. Mitochondria also generate ROS and regulate the cell death pathways, and therefore, the importance of the mitochondrial role in cancer is under reassessment [32]. Moreover, some works point towards a metabolic reprogramming induced by leptin consisting of an enhanced use of lipids as metabolic fuel, preventing the accumulation of lipids in non-adipose tissues (lipotoxicity) [33]. The

enhanced use of fatty acids also involves functional mitochondria, since β -oxidation pathway highly depends on functional oxidative phosphorylation.

Therefore, in highly proliferative cancer cells ROS regulation is crucial owing to the presence of oncogenic mutations that promote aberrant metabolism with increased rates of ROS production, leading to cell death [34]. Given that leptin has the ability to influence cellular metabolism and activate multiple oncogenic pathways, our aim was to study whether leptin-mediated metabolic alterations may affect ROS regulation, which may contribute to breast cancer pathogenesis in obese postmenopausal women. Moreover, we analyzed the relevance of hyperactive leptin-signalling network in CDDP chemotherapy by studying cancer cell viability after leptin exposure.

Materials and Methods

Reagents

Dulbecco's Modified Eagle's medium (DMEM) high glucose was from GIBCO (Paisley, UK). Leptin, genipin, cisplatin (cis-diamminedichloroplatinum II or CDDP) and *tert*-Butyl hydroperoxide (t-BOOH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Routine chemicals used were obtained from Sigma-Aldrich, Roche (Barcelona, Spain), Panreac (Barcelona, Spain) and Bio-Rad Laboratories (Hercules, CA, USA).

Cell culture and treatment

MCF-7 human breast cancer cell line was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and routinely cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotics (penicillin and streptomycin) in a humidified atmosphere of 5% CO₂ at 37 °C (control cells). For chronic leptin treatment, cells were cultured with fresh medium supplemented with leptin 100 ng/mL for 10 days (leptin cells). The concentrations of leptin required to induce cell proliferation *in vitro* were 25–100 ng/ml [35]. All experiments were carried under subconfluence conditions.

Cell proliferation assay

Control and leptin MCF-7 cells were seeded in 96-well plates, incubated overnight, and treated with 75 μ M genipin, 10 μ M CDDP or 25 μ M t-BOOH for 24h. At the end of the treatments, cells were stained with 0.5% (p/v) crystal violet in 30% (v/v) acetic acid for 10 min. After washing, the dye was solubilized with 100 μ l of methanol and absorbance was measured photometrically (A595nm) with a PowerWave XS Microplate Spectrophotometer (BioTek Instruments, Inc.) to determine cell growth.

Fluorometric determination of intracellular ROS

The ROS levels were measured fluorimetrically following the manufacture protocol of Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Eugene, Oregon). Both control and leptin MCF-7 cells were seeded in 96-well plates, incubated overnight, and treated with 75 μ M genipin and 10 μ M CDDP for 24h. The reaction mixture, which contains 50 μ M Amplex Red reagent and 0.1 U/ml horseradish peroxidase in Krebs-Ringer phosphate, was added to cells. Fluorescence measurement was performed in a FLx800 microplate fluorescence reader (Bio-Tek Winooski, Vermont, USA) set at excitation and emission wavelengths of 571 and 585 nm, respectively. The values were normalized per number of viable cells determined by crystal violet assay.

Western blot analysis

Cells were harvested by scraping in lysis buffer (20 mM Tris-HCl, 1.5 mM MgCl₂, 140 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, 1 mM EGTA, 1 mM Na₂VO₄, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/mL leupeptin and 10 μ g/mL pepstatin; pH 7.4) and disrupted by sonication. Afterwards, protein content was determined with a bicinchoninic acid protein assay kit (Pierce, Bonn, Germany). 40 μ g of cell lysate protein was separated on a 12% SDS-PAGE gel and electrotransferred onto nitrocellulose membranes. Membranes were incubated in a blocking solution of 5% non-fat powdered milk in Tris-buffered saline-Tween (TBS with 0.05% Tween-20). Antisera against UCP2, PRX III and α -Tubulin (Santa Cruz Biotechnology, CA, USA); OXPHOS Complex (MitoSciences, OR, USA); Catalase, CuZn-SOD and Mn-SOD (Calbiochem, CA, USA); and

SIRT1 (Millipore, CA, USA) were used as primary antibodies. Protein bands were visualized by Immun-Star® Western C® Kit reagent (Bio-Rad) Western blotting detection systems. The chemiluminescence signal was captured with a Chemidoc XRS densitometer (Bio-Rad) and results were analyzed with Quantity One Software (Bio-Rad).

Measurement of 4-HNE adducts and carbonyl contents

For 4-hydroxy-2-nonenal (4-HNE) and carbonyl groups analysis, 40 µg and 10 µg of protein from cell lysate, respectively, were separated on a 12% SDS-PAGE gel and electrotransferred onto nitrocellulose membrane. Protein carbonyls were detected using the immunological method OxySelect™ Protein Carbonyl Immunoblot kit (Cell Biolabs, San Diego, CA, USA). For carbonyl group derivatization, the membrane was incubated with 2, 4-dinitrophenylhydrazine (DNPH) for 5 min, and then processed according to the manufacturer's instructions. Unspecific binding sites on the membranes were blocked in 5% non-fat milk in TBS-T (Tris-buffered saline-with 0.05% Tween-20). Antiserum against 4-HNE (Alpha Diagnostic International, San Antonio, TX, USA) and DNP were used as primary antibodies. Bands were visualized using the Immun-Star® Western C® Chemiluminescent Kit (Bio-Rad). The chemiluminescence signal was captured with a Chemidoc XRS densitometer (Bio-Rad Laboratories) and results were analyzed with Quantity One software (Bio-Rad Laboratories).

Statistics

The Statistical Program for the Social Sciences software for Windows (SPSS, version 21.0; SPSS Inc, Chicago, IL) was used for all statistical analyses. Data are expressed as means ± standard error of the mean (SEM) of six independent biological replicates. Differences between control and leptin cells were assessed by Student's t-test. The effects of leptin and drugs (CDDP, t-BOOH and genipin) were evaluated by two-way analysis of variance (ANOVA). Least significant difference (LSD) test was used as a post hoc comparison. Statistical significance was set at $P < 0.05$.

Results

Effect of chronic leptin on MCF-7 cell growth and CDDP cytotoxicity

It is well known that leptin is a potent mitogen in breast cancer cell lines, including MCF-7 [10, 36]. To confirm this effect in our chronic-leptin model (10 days), cell proliferation was analyzed. Similar to previous works [9, 37], chronic-leptin increased cell proliferation by 30% (Table 1). Next, we examined if chronic exposure to leptin may compromise the effectiveness of CDDP, a chemotherapy drug able to induce ROS formation. As expected, CDDP decreased cell proliferation, with this decrease less marked in leptin cells than in control cells. As shown in Figure 1A, leptin increased by 37% the tolerance of MCF-7 to CDDP treatment.

Table 1. Effect of chronic leptin on cell proliferation, ROS levels and oxidative damage parameters in MCF-7 cells. Data represent the means ± SEM (n=6). Values of control (non-treated) MCF-7 cell line were set at 100. AU: arbitrary units; 4-HNE: 4-hydroxy-2-nonenal; ROS: reactive oxygen species. *Significant difference between leptin-treated and non-treated cells (Student's t-test; $P < 0.05$)

	MCF-7					
	Control			Leptin		
Proliferation levels (AU)	100	±	1	130	±	1*
ROS levels (AU)	100	±	4	71.1	±	6.1*
4-HNE adducts content (AU)	100	±	8	83.3	±	8.0
Carbonyl groups content (AU)	100	±	8	62.0	±	4.7*

Fig. 1. Effect of chronic leptin on MCF-7 cell growth and CDDP or t-BOOH cytotoxicity. A) Cell viability response to CDDP treatment in leptin cells. Cells were maintained in the media supplemented with leptin 100 ng/mL for 10 days. At 9th day of leptin exposure, cells were treated with CDDP 10 μ M. Cell proliferation was analyzed by Crystal Violet assay as described in Materials and Methods. Data represent the means $\pm$ SEM (n=6), are normalized as percentage of the control value (non-treated: 0 ng/mL leptin and without CDDP treatment). B) Cell viability response to t-BOOH treatment in leptin cells. At 9th day of leptin exposure, cells were treated with t-BOOH 25 μ M. Data represent the means $\pm$ SEM (n=6), are normalized as percentage of the control value (non-treated: 0 ng/mL leptin and without t-BOOH treatment). Two-way ANOVA (P<0.05), L: leptin effect; T: treatment effect, T*L: interactive effect. Post hoc analysis LSD (P<0.05): *CDDP or t-BOOH treated vs non-treated; °Leptin-treated vs control.

Fig. 2. Chronic leptin exposure attenuates the CDDP-induced ROS production. MCF-7 cells were maintained in media supplemented with or without 100 ng/mL leptin for 10 days and 24h before ROS analysis cells were treated with 10 μ M CDDP. Data represent the means $\pm$ SEM (n=6), normalized as percentage of the control value (non-treated: 0 ng/mL leptin and without CDDP treatment). Two-way ANOVA (P<0.05), L: leptin effect; T: treatment effect; T*L: interactive effect. Post hoc analysis LSD (P<0.05): *CDDP or t-BOOH treated vs non-treated; °Leptin-treated vs control.

In order to check whether leptin cells are more resistant to oxidative stress, we treated the cells with t-BOOH for 24h. Results show a slightly higher tolerance to t-BOOH with leptin (14%) (see Fig.1B).

Chronic leptin exposure decrease ROS production

We examined whether chronic leptin was able to alter ROS production in MCF-7 cells. As shown in Table 1, leptin treatment decreased ROS levels by 28%. In addition, to evaluate whether chronic leptin treatment could affect the efficacy of cytotoxic CDDP, we determined the ROS levels. As shown in Figure 2, leptin treatment decreased ROS levels by 35% in CDDP-treated cells compared to the control cells with the same treatment, concluding that chronic leptin stimulation results in a decrease of ROS production.

Decrease in carbonyl content after chronic leptin treatment

ROS are able to damage all types of biological molecules. Hence, we studied levels of carbonyl groups and 4-HNE adducts in proteins (end markers of oxidative damage in proteins and lipids, respectively) in leptin cells. Our findings indicate that chronic leptin treatment significantly decreased carbonyl content (40%) and a similar trend was observed for 4-HNE adduct formation, although the latter did not reach statistical significance (P=0.085) (Table 1).

Table 2. OXPHOS complexes protein levels in MCF-7 breast cancer cell line after leptin treatment. Data represent the means $\pm$ SEM (n=6). Values of control (non-treated) MCF-7 cell line were set at 100. AU: arbitrary units; Complex V: ATP synthase subunit alpha – “CV-alpha”; Complex III subunit core 2 – “CIII-core2”; Complex II-FeS subunit 30 kDa – “CII-30”; Complex IV subunit II – “CIV-II”; Complex I subunit NDUFB8 – “CI-20”. *Significant difference between leptin-treated and non-treated cells (Student’s t-test; $P < 0.05$)

	MCF-7	
	Control	Leptin
Complex V (AU)	100 $\pm$ 10	105 $\pm$ 12
Complex III (AU)	100 $\pm$ 9	113 $\pm$ 15
Complex II (AU)	100 $\pm$ 10	112 $\pm$ 8
Complex IV (AU)	100 $\pm$ 5	105 $\pm$ 15
Complex I (AU)	100 $\pm$ 4	76.1 $\pm$ 9.3*

Fig. 3. Representative bands of OXPHOS complex and Tubulin. Data have been represented in Table 2 as the means $\pm$ SEM (n=6). Complex V: ATP synthase subunit alpha – “CV-alpha”; Complex III subunit core 2 – “CIII-core2”; Complex II-FeS subunit 30 kDa – “CII-30”; Complex IV subunit II – “CIV-II”; Complex I subunit NDUFB8 – “CI-20”. Tubulin was used as a housekeeping protein.

Fig. 4. Effect of leptin on genipin-induced ROS levels in MCF-7 cells. Cells were maintained in the media supplemented with leptin 100 ng/mL for 10 days. At 9th day of leptin exposure, cells were treated with genipin 75 μ M. Data represent the means $\pm$ SEM (n=6), are normalized as percentage of the control value (non-treated: 0 ng/mL leptin and without genipin treatment). Two-way ANOVA ($P < 0.05$), L: leptin effect; T: treatment effect; T*L: interactive effect. Post hoc analysis LSD ($P < 0.05$): *Genipin-treated vs non-treated, °Leptin-treated vs control.

Effect of chronic leptin treatment on mitochondria respiratory complex and UCP2 expression levels

The main source of ROS is the mitochondria, which in turn, are the first target of harmful ROS effects [38]. Specifically, ROS are produced by the electron transport chain, where respiratory complexes I and III are the major points of electron leak and lead to superoxide formation [39]. To test whether chronic-leptin may decrease ROS formation by altering OXPHOS complex, we analyzed their levels by Western blot. Our results indicate that leptin did not produce any changes in mitochondrial OXPHOS complexes, except for a decrease (24%) in Complex I (NADH: ubiquinone oxidoreductase) (see Table 2 and Fig. 3).

On the other hand, we studied uncoupling protein 2 (UCP2), membrane protein that is able to uncouple respiration from ATP synthesis and prevent ROS production. UCP2 protein levels decreased by 26% in cells exposed to leptin (Table 3). Moreover, to check this effect

Table 3. Response of antioxidant enzymes and UCP2 protein levels in MCF-7 cells chronically exposed to leptin. Data represent the means $\pm$ SEM (n=6). Values of control (non-treated) MCF-7 cell line were set at 100. AU: arbitrary units; CuZn-SOD: copper and zinc superoxide dismutase; Mn-SOD: manganese superoxide dismutase; PRX III: Peroxiredoxin III; UCP2: uncoupling protein 2. *Significant difference between leptin-treated and non-treated cells (Student's t-test; $P < 0.05$)

	MCF-7	
	Control	Leptin
PRX III levels (AU)	100 $\pm$ 7	102 $\pm$ 17
Catalase levels (AU)	100 $\pm$ 6	118 $\pm$ 8
CuZn-SOD levels (AU)	100 $\pm$ 11	92.4 $\pm$ 10.7
Mn-SOD levels (AU)	100 $\pm$ 7	94.4 $\pm$ 6.6
UCP2 levels (AU)	100 $\pm$ 9	74.0 $\pm$ 10.6*

Fig. 5. Chronic Leptin exposure enhances the expression of Sirtuin1. Protein expression was measured in MCF-7 cells treated with or without 100 ng/mL leptin for 10 days by Western blotting using a specific antibody against SIRT1. Bars represent the mean $\pm$ SEM (n=6) of band intensity expressed as percentage of control cells. *Significant difference between leptin-treated and non-treated cells (Student's t-test; $P < 0.05$).

of leptin on UCP2 functionality, we inhibited this protein with genipin and determined ROS levels. Leptin cells treated with genipin showed lower rise in ROS levels than control cells (37%), indicating a lower UCP2 activity with leptin (Fig. 4).

Effects of chronic leptin treatment on antioxidant enzymes and SIRT1 expression levels

The balance between ROS production and the scavenging by antioxidant defenses determines oxidative stress. Thus, the protein levels of main antioxidant enzymes were evaluated (Mn-SOD, CuZn-SOD, Catalase and PRX III). Our data showed that leptin did not have any effect on antioxidant enzymes (Table 3). Finally, we measured the levels of SIRT1, one of the main factors involved in adaptive response against oxidative stress [40, 41]. The SIRT1 protein levels increased by 60% in chronically leptin treated cells (Fig. 5).

Discussion

Epidemiological evidences supports a correlation between obesity and breast cancer in postmenopausal women [1]. Leptin, a hormone excessively produced during obesity, has been suggested to be involved in breast cancer [7, 8]. Recent investigations have reported that this adipokine stimulates a proliferative response in normal and cancerous mammary epithelial cells [10]. In accordance, the present study shows that chronic leptin induces cell growth and decreases oxidative stress in MCF-7 breast cancer cells, with slight changes in OXPHOS complexes and UCP2 protein levels, and more significant ones in SIRT1. The regulation of oxidative stress is an important factor in both tumor development and response to anticancer therapies [17]. In fact, redox status of cancer cells usually differs from that of normal cells, since cancer cells exhibit elevated ROS levels due to metabolic alterations [34]. In our study, leptin increased the tolerance in MCF-7 cells to drug (CDDP)-mediated ROS production.

Our results showed that chronic leptin treatment decreases ROS levels in MCF-7 cells. It is well known that ROS can lead to protein and lipid oxidation [42] and, consistently, we found that leptin decreased oxidative damage, which supports the hypothesis that this adipokine could protect against oxidative stress. Consistent with our findings, Ho PW et

al. reported that leptin preserved cell survival in neuronal cells against MPP⁺ (1-methyl-4-phenylpyridinium) toxicity [43]. However, it is to be noted that obesity is associated with oxidative stress as a result of inflammatory and metabolic changes [44]. Ho PW et al. described induction of UCP2 expression by leptin, indicating that UCP2 was critical in mediating the neuroprotective effects of leptin against MPP⁺ toxicity [43]. However, in this current work, we detected a decrease in both ROS and UCP2 protein levels after leptin treatment. Furthermore, our results were confirmed by a functional assay with genipin, a specific inhibitor of UCP2. The inhibition of UCP2 activity by genipin results in a marked increase in the rate of mitochondrial ROS production [45]. This increase was significantly lower in leptin treated cells, which confirms the ability of leptin to decrease UCP2 activity. UCP2 is a key protein regulator of stress response and metabolism [46], and several studies have shown that the antioxidant UCP2 is widely over-expressed in cancer cells, providing support to the view that this feature as an adaptive mechanism developed by tumors to maintain ROS homeostasis [47, 48]. However, it is worthy to note that UCP2 acts as a sensor of mitochondrial oxidative stress and is activated by ROS [49]. Particularly, some reports have demonstrated that the UCP2-mediated proton leak is activated by mitochondrial superoxide and by the formation of lipid peroxidation derivatives such 4-hydroxynonenal [50]. In agreement with our results, we could deduce that the decrease in UCP2 levels is more a consequence than a cause for the lower ROS in leptin treated cells. Similarly, antioxidant enzymes protein levels were not induced by leptin. Nevertheless, the relationship between leptin and oxidative stress is controversial. In contrast to ability of leptin to reduce oxidative stress discussed above, the opposite effect has been reported, showing a strong association between obesity and oxidative stress in relation to inflammation and metabolic syndrome [44, 51].

In an effort to understand the effects of chronic leptin on oxidative stress, we analyzed the main source of ROS: mitochondrion. ROS are generated during mitochondrial oxidative metabolism [52], therefore the lower levels could be due to a lower production by mitochondria and/ or a higher energy demand owing to the higher cell growth. Regarding mitochondria status, all mitochondrial respiratory chain complexes remained unchanged, except for Complex I, one of the main contributors to superoxide production by mitochondria [39], and this Complex was significantly decreased by leptin. Lower Complex I levels could contribute to decrease oxidative damage in these cells. Alternatively, the higher proliferation rate induced by leptin may increase the ATP demand and, therefore, electron transfers couple to ATP production and reduce the proton-motive force, with a subsequent decrease in the rate of ROS production [34]. According to this hypothesis, our recent published data show the ability of leptin to improve mitochondrial function by increased bioenergetic efficiency and a low ROS production, conferring benefits for growth and survival to MCF-7 breast cancer cells [53]. On the other hand, the cell content of the Complex I could be associated with a shift in metabolic substrate from glucose to fatty acids utilization. In fact, several studies have demonstrated increased lipolysis in cancer, suggesting the use of fatty acids as fuels [33]. In addition, studies in muscle have found that leptin activates AMPK signalling pathway, stimulating fatty acid oxidation [54, 55]. Therefore, leptin-AMPK signalling pathway may be conserved and activated in breast cancer cells and be underlying the adaptations of energy metabolism for cell growth.

Prevention of intracellular ROS is arguably more efficient than the elimination of already formed reactive radicals. In the current work, we observed that leptin enhanced SIRT1 protein levels, which sense changes in intracellular NAD⁺ levels that reflect energetic state of cell [56]. SIRT1 is one of the main factors involved in adaptive response against oxidative stress and improving energy efficiency [41, 57], and allows the cell to adapt to situations of energy stress. In fact, studies in SIRT1-null mice reported that liver mitochondria were metabolically inefficient compared to their normal controls [58]. Furthermore, it has been proposed that UCP2 is negatively regulated by SIRT1 [59], and this observation is consistent with our results which show that the ability of leptin to down-regulate UCP2, which allows a better coupling and ATP production in response to high energetic requirements for cell

growth. Nevertheless, there are other regulators of mitochondrial activity that should be further investigated to determine whether leptin could also modulate them [60]. For instance, some authors have reported that leptin increases mitochondrial biogenesis through PGC-1 α activation in skeletal muscle and adipose tissue [33, 54], and recently in breast cancer cells [53]. AMPK and SIRT1 directly affect PGC-1 α activity through phosphorylation and deacetylation, respectively [61]. This mechanism would allow leptin cell to increase mitochondrial respiration and meet the energetic requirements of the cell in circumstances of energy stress. Indeed, LeBleu et al. have found that PGC-1 α enhanced mitochondrial biogenesis and oxidative phosphorylation in invasive cancer cells, promoting metastasis [62].

One of the major factors in the failure of chemotherapy is the development of drug resistance [63]. In addition to mitogenic effects, in this study, leptin showed the ability to increase the resistance of cancer cells to CDDP. CDDP is one of the most powerful chemotherapy drug whose antitumor action is via DNA alkylation [23]. Nevertheless, recent evidences have reported the ability of this anticancer agent to interfere with cell metabolism and how the resulting redox imbalance is one of the main mechanisms underlying toxicity of CDDP [24, 64]. As shown in this study, CDDP treatment induces a large ROS formation, leading to higher oxidative stress. However, cells exposed to leptin showed a higher tolerance to the CDDP cytotoxic effect, thus ROS formation induced by this drug was lower in leptin cells than in control cells. In this regard, overexpressed SIRT1 in leptin cells might result in a higher threshold for ROS mediated-apoptosis and explain the resistance to CDDP after leptin exposure. In fact, some works have demonstrated that SIRT1 upregulation contributes to CDDP resistance in association with altered mitochondrial metabolism [65, 66]. On the other hand, previous studies from our laboratory and others demonstrated that leptin-signalling pathway induces phosphorylation of STAT3 [9], and it is widely accepted that constitutive activation of the STAT3 pathway confers resistance to CDDP-induced apoptosis in ovarian cancer cells [67, 68]. Therefore, leptin may desensitize breast cancer cells to CDDP antitumor action through STAT3 activation. In addition, leptin increased the resistance to another oxidative stress inducer, t-BOOH. On the basis of the results found, leptin could be advantageous for cancer cells, at least partially, protecting them from oxidative stress.

Most epidemiological studies show that leptin pathway may be involved in breast cancer, and it would explain, in part, the positive correlation between obesity and breast cancer in postmenopausal women, as well as the association of hyperleptinemia with a worse pathology prognosis. Consistent with our findings, by decreasing basal oxidative stress, leptin may protect the malignant cells from oxidative status of breast tumor environment and promote their survival and the resistance to oxidative stress promoting agents, such as cisplatin. Future clinical trials are warranted to define the significance of leptin as prognosis factor for the effectiveness in treatment of breast cancer patients, and to evaluate its role in cellular energy metabolism and oxidative stress regulation.

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Disclosure Statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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