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Leptin Modulates Mitochondrial Function, Dynamics and Biogenesis in MCF-7 Cells

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ABSTRACT

The adipokine leptin, known for its key role in the control of energy metabolism, has been shown to be involved in both normal and tumoral mammary growth. One of the hallmarks of cancer is an alteration of tumor metabolism since cancerous cells must rewire metabolism to satisfy the demands of growth and proliferation. Considering the sensibility of breast cancer cells to leptin, the objective of this study was to explore the effects of this adipokine on their metabolism. To this aim, we treated the MCF-7 breast cancer cell line with 50 ng/mL leptin and analyzed several features related to cellular and mitochondrial metabolism. As a result, leptin increased cell proliferation, shifted ATP production from glycolysis to mitochondria and decreased the levels of the glycolytic end-product lactate. We observed an improvement in ADP-dependent oxygen consumption and an amelioration of oxidative stress without changes in total mitochondrial mass or specific oxidative phosphorylation (OXPHOS) complexes. Furthermore, RT-PCR and western blot showed an up-regulation for genes and proteins related to biogenesis and mitochondrial dynamics. This expression signature, together with an increased mitophagy observed by confocal microscopy suggests that leptin may improve mitochondrial quality and function. Taken together, our results propose that leptin may improve bioenergetic efficiency by avoiding the production of reactive oxygen species (ROS) and conferring benefits for growth and survival of MCF-7 breast cancer cells. J. Cell. Biochem. 116: 2039–2048, 2015.

KEY WORDS: BREAST CANCER; LEPTIN; MITOCHONDRIA; BIOENERGETIC; BIOGENESIS; FISSION; FUSION; MITOPHAGY

Leptin is a 167-aminoacid peptide hormone (16 kDa) product of Ob gene, mainly synthesized by adipocytes and to a lesser extent by other tissues such as stomach, placenta, muscle, immune cells, and mammary gland [Schubring et al., 1999; Ahima and Flier, 2000]. Leptin concentration in blood rises as body weight and fat mass increase [Sweeney, 2002] and regulates energy homeostasis by suppressing food intake and increasing energy expenditure by acting on transmembrane Ob-R receptors located in hypothalamic nuclei [Cohen, 2006]. From studies performed to date, it is known that Ob-R receptors are expressed, in addition to central nervous system, in many other tissues including islet cells, liver, kidney, lung, skeletal muscle, bone marrow, and breast, among others [Margetic et al., 2002; Bjorbaek and Kahn, 2004]. In these peripheral tissues, leptin has been shown to influence multiple functions including fetal development, sex maturation,

Abbreviations: 2-DG, 2-deoxyglucose; AMPK, 5′ AMP-activated protein kinase; pAMPK, phosphorylated 5′ AMP-activated protein kinase; COX, cytochrome C oxidase; DCFDA, 2′, 7′-dichlorofluorescein diacetate; DNPH, 2, 4-dinitrophenylhydrazine; DRP1, dynamin-related protein-1; ETC, electron transport chain; FIS1, mitochondrial fission protein 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LTR, lysotracker red; MDC, monodansylcadaverine; MFN1, mitofusin-1; MFN2, mitofusin-2; MTG, mitotrackergreen; MTSSB, mitochondrial single strand DNA binding protein; OCR, oxygen consumption rate; OPA1, optic atrophy-1; OXPHOS, oxidative phosphorylation; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; ROS, reactive oxygen species; TFAM, mitochondrial transcription factor.

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lactation, hematopoiesis, and immune responses [Grossmann and Cleary, 2012]. In mammary gland, leptin has been shown to be a mitogenic factor necessary for normal development [Hu et al., 2002].

In previous studies, it has been reported that leptin increases cell proliferation and inhibits apoptosis in several human cell lines, including breast [Nakao et al., 1998; Konopleva et al., 1999; Saxena et al., 2007; Liu et al., 2011; Han et al., 2012]. In fact, it has been found that Ob-R receptor is present in human breast cancer cells [Grossmann and Cleary, 2012], and both leptin and its receptor are overexpressed in invasive ductal carcinomas [Ishikawa et al., 2004]. High levels of serum leptin, together with a high Ob-R expression in the tumor, have been pointed to be a poor prognosis predictor in patients with breast cancer [Miyoshi et al., 2006]. Hence, the hyperleptinemia associated with obesity is currently a subject of intense investigation since obesity is a known risk factor for breast cancer in postmenopausal women [Cleary and Maihle, 1997; Willett, 1997; Calle and Thun, 2004].

Cancer cells must rewire cellular metabolism to satisfy the demands of growth and proliferation. As commonly accepted, most tumor cells rely on their ATP synthesis by glycolysis rather than on oxidative phosphorylation, a phenomenon known as the Warburg effect [Warburg, 1956]. Despite this fact was initially attributed to mitochondrial dysfunction, this hypothesis is now being re-evaluated due to the important role of this organelle in cancer. In fact, in cancerous states, anaplerotic and cataplerotic mitochondrial reactions work together to provide enough biosynthetic precursors to sustain growth and proliferation. Thus, in contrast to Warburg's first observations, maintaining functional mitochondria appears to be key for cancer cell survival and proliferation [Wallace, 2012]. Mitochondria are dynamic organelles that continually undergo fusion and fission. These apparently opposite processes, termed mitochondrial dynamics, work in concert to maintain the shape, size, number, and function of mitochondrion [Chan, 2012]. Furthermore, mitochondrial biogenesis and mitophagy represent two co-ordinated processes that also determine mitochondrial content, structure, and function [Zhu et al., 2013].

To date, little effort has been made to study the link between leptin and metabolism in breast cancer cells. There is one previous report in the literature showing that mice deficient in the peripheral leptin receptor have an ATP production less reliant on glycolysis and an increased capacity for β-oxidation [Park et al., 2010]. In contrast to this observation in breast, other authors have found that leptin stimulates fatty acid oxidation, glucose uptake and ROS production in muscle, endothelial cells and adipocytes [Yamagishi et al., 2001; Minokoshi et al., 2002; Luo et al., 2008], thus activating mitochondrial function [Henry et al., 2011].

Taking into account the sensibility of breast cancer to leptin and the lack of studies exploring its metabolic effects in this tissue, our goal was to analyze the effects of a physiological dose of this hormone in several features of cellular and mitochondrial metabolism in the widely used MCF-7 breast cancer cell line.

MATERIALS AND METHODS

REAGENTS

Dulbecco's modified Eagle's medium (DMEM) high glucose was from GIBCO (Paisley, UK). Fetal bovine serum (FBS) and penicillin-streptomycin were from Biological Industries (Kibbutz Beit Haemek, Israel). Hoechst 33342, 2′, 7′-dichlorofluorescein diacetate (DCFDA), 2-deoxyglucose (2-DG), oligomycin, antimycin A, monodansylcadaverine (MDC), and leptin were from Sigma-Aldrich (St. Louis, MO). ApoSensor™ Cell Viability Assay Kit was purchased from BioVision (Milpitas, CA), TriPure® isolation reagent from Roche (Barcelona, Spain), primers used were from Metabion (Germany), BCA protein assay from Pierce (Bonn, Germany), and Total OXPHOS human WB antibody cocktail (#MS601) from MitoSciences (Eugene, OR). TFAM (SC-30962), MFN2 (SC-50331), DRP1 (SC-32898), total AMPKα (SC-25792), phospho-AMPKα (SC-33524), and GAPDH (sc-25778) human WB antibodies were from Santa Cruz Biotechnology (Texas), Immun-Star© Western C© Chemiluminescent Kit from Bio Rad Laboratories (Hercules, CA), and OxySelect™ Protein Carbonyl Immunoblot kit was from Cell Biolabs (San Diego, CA). Finally, Lysotracker Red DND-99 (LTR) and Mitotracker Green (MTG) were from Invitrogen-Molecular Probes.

CELL CULTURE AND PROLIFERATION ASSAY

MCF-7 breast cancer cell line was purchased from American Type Culture Collection (ATCC; Manassas, VA) and cultured in DMEM supplemented with 10% FBS and 1% antibiotic (penicillin and streptomycin) at 37°C in 5% CO_2. Cells were treated when cultures reached 80% confluence by providing fresh medium supplemented with 50 ng/mL leptin or without (control cells). For proliferation assay, cells were seeded in 96-well plates (7,500 per well) and leptin-treated for 24 h; next, cell proliferation was determined by staining DNA with Hoechst 33342 at a concentration of 0.01 mg/mL for 5 min at 37°C. Cell culture medium was shifted to PBS and fluorescence was measured in a FLx800 microplate fluorescence reader (BIO-TEK Winooski, VT) set at 360 nm excitation and 460 nm emission wavelengths.

INTRACELLULAR ATP LEVELS

For measurement of ATP levels after glycolysis or ATP synthase inhibition, cells were seeded in 96-well plates and cultured for 24 h with leptin. Next, 10 mM 2-DG, 1 μM oligomycin (glycolysis and mitochondrial ATP synthase inhibitors, respectively) or vehicle were added and incubated for 30 min and ATP levels were measured using the ApoSensor™ Cell Viability Assay Kit. Cell number was determined by the crystal violet method [Nagamine et al., 2009], in parallel-treated wells, in order to normalize ATP levels. Briefly, 20 μL of Violet Crystal solution (0.5% of Violet Crystal in 30% acetic acid) were added to each well and incubated for 10 min at room temperature. The medium was removed and the plate was washed twice with water. Finally, to solubilize the dye, 100 μL of methanol were added and plate was shaken for 1 min. Absorbance was measured at 570 nm using a microplate reader (Power Wave XS, BIO-TEK).

LACTATE PRODUCTION

Cells were seeded in a 96-well plates and treated with leptin 50 ng/mL for 24 and 48 h. After treatment, media was replaced by fresh media supplemented or not with leptin and 10 mM 2-DG, 1 μM oligomycin or vehicle. Within the next 8 h, aliquots of media were collected to determine lactate production by an enzymatic assay. Briefly, lactate was converted into pyruvate in presence of NAD^+ by

lactate dehydrogenase and NADH formation measured by monitoring the fluorescence in a microplate fluorescence reader (BIO-TEK Winooski, VT) set at 360 nm excitation and 460 nm emission. Immediately after collecting media, cell density was determined by the crystal violet method as described previously.

REAL-TIME QUANTITATIVE PCR

After 6 h of treatment with leptin 50 ng/mL, cell culture medium was removed and total RNA was isolated using TriPure® isolation reagent following the manufacturer's protocol and quantified using a spectrophotometer (BioSpec-nano Shimadzu-Biotech) set at 260 nm. One microgram of the total RNA was reverse transcribed to cDNA, and PCR was done for target genes: dynamin-related protein-1 (*DRP1*), mitochondrial fission protein 1 (*FIS1*), mitofusin-1 (*MFN1*), mitofusin-2 (*MFN2*), mitochondrial single strand DNA binding protein (*MTSSB*), optic atrophy-1 (*OPA1*), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PGC-1α*), mitochondrial transcription factor (*TFAM*), and 18S (such as housekeeping), using specific primers (see Table 1) with SYBR Green technology on a LightCycler 480 System II rapid thermal cycler (Roche Diagnostics, Basel, Switzerland). The amplification program consisted of a preincubation step for denaturation of the template cDNA (5 min, 95°C), followed by 45 cycles consisting of denaturation (10 s, 95°C), annealing (10 s, T is detailed in Table 1), and extension steps (12 s, 72°C min). Ct values of the real-time PCR were calculated using the LightCycler Software 3.5.3 by the Second Derivative Maximum method. Ct values were analyzed taking into account the efficiency of the reaction and referring these results to the 18S using the GenEx Standard Software (Multi-DAnalises, Sweden).

WESTERN BLOT

MCF-7 cells were treated with leptin and lysates were prepared by scraping cells in lysis buffer (20 mM Tris–HCl, 1.5 mM $MgCl_2$, 140 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, and 10 μg/mL pepstatin; pH 7.4) and disrupted by sonication. Protein content was determined with a BCA protein assay kit. For Western blot analysis, 35 μg of protein from cell lysates were separated on a 15% 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-20 (TBS with 0.05% Tween-20). Total OXPHOS antibody cocktail, TFAM, MFN2, DRP1, total AMPKα, phospho-AMPKα, and GAPDH (housekeeping) antibodies were used as primary antibodies. Protein bands were visualized by Immun-Star® Western C® Chemiluminescent Kit Western blotting detection systems. The chemiluminescence signal was acquired with a Chemidoc XRS densitometer (Bio-Rad Laboratories) and results were analyzed with Quantity One Software (Bio-Rad).

ENZYMATIC ACTIVITIES

Cells treated with leptin for 24 h were harvested by scraping them out with PBS buffer and then were centrifuged at 2,500 g for 5 min at 4°C to remove cell debris. The resultant cell pellet was resuspended in sterile water and the lysates were kept on ice and protein measured as previously described. ATP synthase (complex V; EC 3.6.1.3) activity was measured by following the oxidation of NADH at 340 nm and 37°C [Ragan et al., 1987] and the extinction coefficient used was 6.22 M/cm. Cytochrome c oxidase (COX) (complex IV or COX; EC 1.9.3.1) was measured using a spectrophotometric method [Chrzanowska-Lightowlers et al., 1993]. Briefly, cell lysate was incubated in 0.1 M $NaPO_4H_2$, pH 7, in the presence of 2 μg/mL catalase and 5 mM substrate DAB (3, 3′-diaminebenzidine tetrachloride). After 30 s, 100 μM reduced cytochrome c was added to start the reaction, and absorbance variation was followed over 15 min at 450 nm.

OXYGEN CONSUMPTION RATE (OCR)

After 24 h with leptin 50 ng/ml, 10^6 cells were harvested and incubated in 1 mL DMEM-FBS in a water-thermostatically

TABLE 1. Primers and Temperature Used for RT-PCR.

Gene	Forward primer (5′–3′) Reverse primer (5′–3′)	T An. (°C)
DRP1	AAgAACCAACCACAggCAAC gTTCACggCATgACCTTTTT	51
FIS1	CTTgCTgTgTCCAAgTCCAA gCTgAAggACgAATCTCAgg	53
MFN1	TTggAgCggAgACTTAgCAT TTCgATCAAgTTCCggATTC	51
MFN2	AgAggCATCAgTgAggTgCT gCAgAACTTTgTCCCAgAgC	56
MTSSB	TgTgAAAAAggggTCTCgAA TggCCAAAgAATCATCC	60
OPA1	ggCCAgCAAgATTAgCTACg ACAATgTCAggCACAATCCA	51
PGC-1α	TCATgCCgTggTAAgTACCA gTgCAAAgTTCCCTCTCTgC	60
TFAM	AgATTggggTCgggTCACT CAAgACAgATgAAAACCACCTC	61
18S	ggACACggACAggATTgACA ACCCACggAATCgAgAAAgA	61

18S, 18S ribosomal RNA; *DRP1*, dynamin-related protein-1; *FIS1*, mitochondrial fission protein 1; *MFN1*, mitofusin-1; *MFN2*, mitofusin-2; *MTSSB*, mitochondrial single strand DNA binding protein; *OPA1*, optic atrophy-1; *PGC-1α*, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; T An., annealing temperature; *TFAM*, mitochondrial transcription factor.

regulated chamber with a computer-controlled Clark-type O_2 electrode (Oxygraph; Hansatech, Norfolk, UK). Cells were pre-incubated for 5 min at 37°C and basal respiration rate was measured three times during the following 5 min. Next, compounds modulating mitochondrial function were added sequentially into the assay medium: oligomycin1 μM was added to reveal ATP synthase-dependent oxygen consumption and antimycin A 1 μM was added to inhibit electron transport chain (ETC) and stop mitochondrial oxygen consumption. Difference between ATP synthase- and ETC-inhibited oxygen consumption was attributed to proton leak.

ROS PRODUCTION ASSAY

ROS production was determined by DCFDA method. Eight-thousand cells were seeded per well in 96-well plates and treated with leptin for 24 h. Plates were incubated for 15 min with 10 μM DCFDA and fluorescence measured in a FLx800 96-well microplate reader (BIO-TEK Winooski, VT) set at excitation and emission wavelengths of 485 and 528 nm, respectively. To normalize ROS production to cell number, culture medium was removed and cell number was measured by staining with Hoechst 33342 as described below.

CARBONYL CONTENT

MCF-7 cells were treated with leptin for 24 h and the presence of carbonyl groups, a measure of protein oxidation, was determined by an immunological method using the OxySelect™ Protein Carbonyl Immunoblot kit. For this purpose, 10 μg of protein from cell lysate were separated on a 12% SDS–PAGE gel and electrotransferred onto nitrocellulose membranes. Protein carbonyls were detected by incubating the membrane with 2, 4-dinitrophenylhydrazine (DNPH) for 5 min. Unspecific binding sites on the membranes were blocked in 5% non-fat milk in Tris-buffered saline-Tween (TBS with 0.05% Tween-20). After incubation with the DNP-antibody, bands were visualized using the Immun-Star© Western C© Chemiluminescent Kit (Bio-Rad) Western blotting detection systems. The chemiluminescence signal was captured with a Chemidoc XRS densitometer (Bio-Rad Laboratories) and results were analyzed with Quantity One software (Bio-Rad Laboratories).

MITOCHONDRIAL, LYSOSOMAL, AND AUTOPHAGOSOMAL MASS

Cells were seeded in 96-well plates and treated with leptin for 24 h. After treatment and in order to determine mitochondrial mass, cells were stained with 0.5 μM mitochondrial specific probe mitotracker

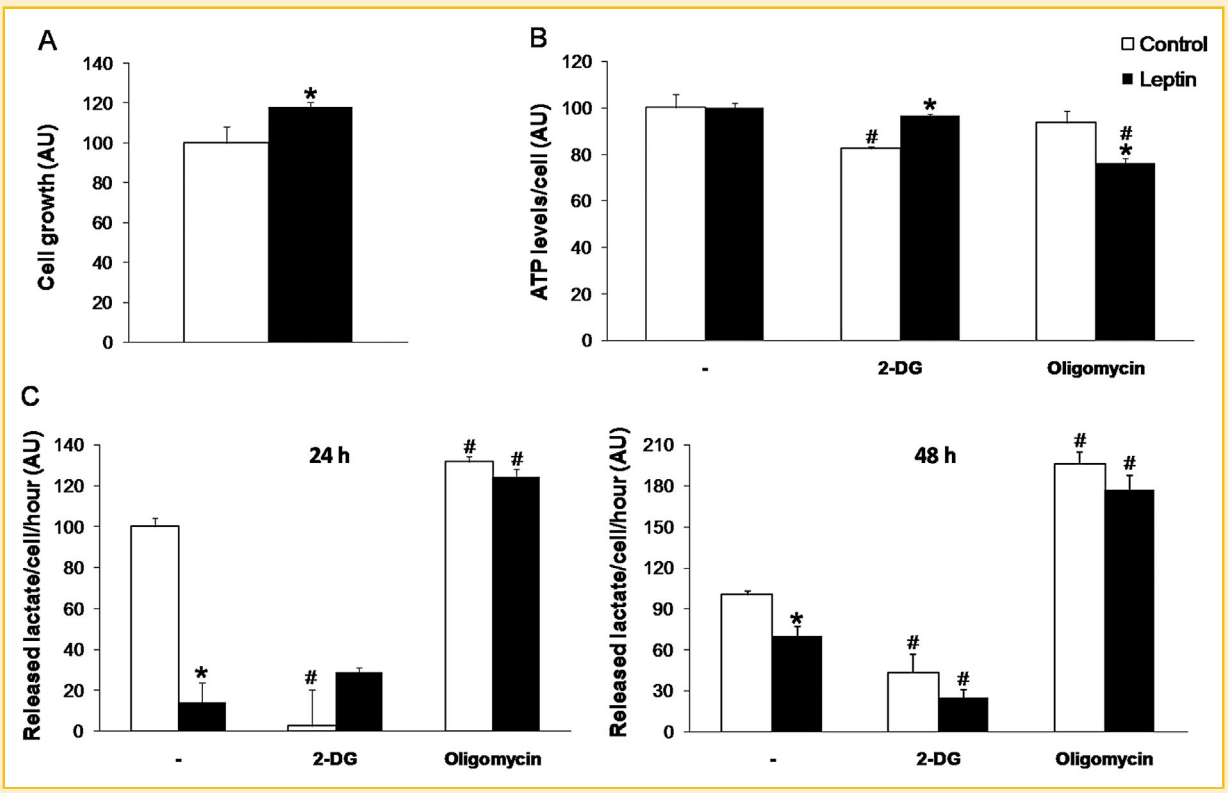


Fig. 1. Cell growth, ATP levels, and released lactate. A) Leptin for 24 h increased cell growth compared to control cells. B) ATP levels were similar between leptin and control cells but leptin increased sensibility to oligomycin (1 μM) and resistance to 2-DG inhibitor (10 mM). C) Leptin-treated cells for 24 h and 48 h presented a lower release of lactate than control cells. Differences between leptin and control cells were annulated after 2-DG or oligomycin addition. Data are expressed in arbitrary units and represent means ± SEM. * Significant difference between leptin-treated and untreated cells (Student's *t*-test; $P \leq 0.05$). # Significant difference between 2-DG or oligomycin-treated and untreated cells (Student's *t*-test; $P \leq 0.05$). 2-DG, 2-deoxyglucose; AU, arbitrary units.

green (MTG) for 60 min. Lysosomes specific probe lysotracker red (LTR) at 77 nM was added for 20 min to evaluate lysosomal mass. To further evaluate the autophagosome formation, after 6 h of leptin treatment cells were incubated with the fluorescent probe monodansylcadaverine (MDC) at 50 μM for 15 min as described by Dando et al. [2013] with modifications. After incubation with the specific probes, culture medium was replaced by PBS and fluorescence was measured in a microplate fluorescence reader (BIO-TEK Winooski, VT) (Ex 485 nm and Em528 nm for MTG, Ex 530 nm and Em590 nm for LTR, Ex 340 nm and Em535 for MDC). The values were normalized per cell density measured by Crystal Violet assay, as described above.

CONFOCAL MICROSCOPY

Cells were co-stained with LTR, MTG, and Hoechst 33342 as described previously by Rodriguez-Enriquez et al. [2009] with some modifications. MCF-7 cells grown in coverslips were stained with 0.5 μM MTG for 60 min at 37°C in culture medium, 20 and 5 min prior to image acquisition 77 nM LTR and 1 μg/mL Hoechst 33342 were added, respectively, to the media. The fluorescence was monitored with a Leica TCS-SP2 confocal microscope and three different fields for each sample were analyzed with *Leica LAS AF* software.

STATISTICAL ANALYSIS

The Statistical Program for the Social Sciences software for Windows (SPSS, version 18.0; SPSS Inc, Chicago, IL) was used for all statistical analyses. Data are presented as means or as linear fold change (in the case of mRNA levels) ± standard error of the mean (SEM). Statistical differences between treated and non-treated cells were analyzed by Student's *t*-test and statistical significance was set at $P < 0.05$. For confocal microscopy images measurements, the tools comprised in the software *Leica LAS AF* were used.

RESULTS AND DISCUSSION

Previous studies have clearly demonstrated that leptin stimulates proliferation of normal and cancerous mammary epithelial cells [Hu et al., 2002; Artac and Altundag, 2012; Grossmann and Cleary, 2012; Guo et al., 2012]. In accordance, 24 h leptin treatment increased cell growth by 18% with respect to untreated MCF-7 cells (Fig. 1A). Considering that cell metabolism reprogramming is needed to sustain this enhanced cell growth in cancerous states, we decided to explore the effects of leptin on MCF-7 metabolism. Cancer cells rely mainly on aerobic glycolysis for ATP production in detriment of oxidative phosphorylation, phenomenon known as Warburg effect

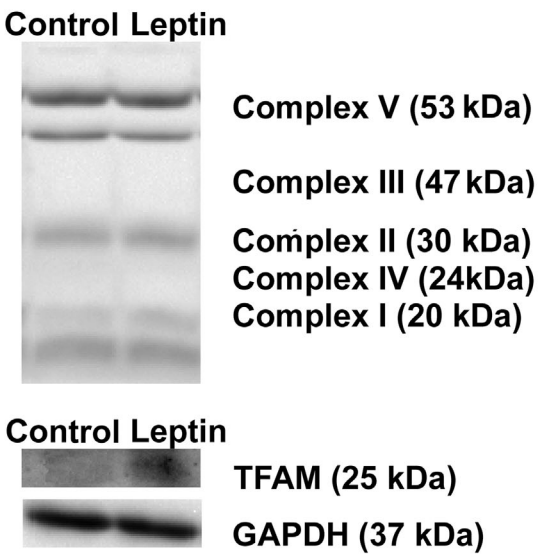
TABLE 2. Parameters Related to Mitochondrial Biogenesis and Bioenergetics. (A) An increased expression of mRNA biogenesis-related markers was observed after 6 h leptin treatment. Data are expressed as linear fold change (leptin vs. control) and positive values indicate up-regulation. The increase in TFAM protein levels also points towards an enhanced biogenesis. No differences were observed in OXPHOS protein levels (48 h leptin) or in maximal ATP synthase or COX activities (24 h leptin), which suggests that mitochondrial mass was not affected. Control values were set at 100, data are expressed in arbitrary units and represent means ± SEM (n = 6). (B) Immunoblots showing representative bands of OXPHOS complexes, TFAM, and GADPH as housekeeping.

A

	mRNA (Linear fold change)
	Leptin vs. control
PGC-1α	1.69 ±0.22*
TFAM	1.11 ±0.10
MTSSB	1.31 ±0.06*

	Protein levels (AU)	
	Control	Leptin
Complex V	100 ±2.22	102 ±8.14
Complex III	100 ±4.02	98.8 ±4.24
Complex II	100 ±9.53	104 ±10.9
Complex IV	100 ±11.7	125 ±33.9
Complex I	100 ±16.7	86.9 ±16.1
TFAM	100 ±8.15	151 ±20.5*
	Maximum activity (AU)	
	Control	Leptin
ATPase	100 ±3.55	104 ±4.50
COX	100 ±16.5	104 ±8.16

B



AU, arbitrary units; complex I, subunit NDUFB8-"CI-20"-20 kDa; complex II, FeS subunit 30 kDa-"CII-30"-30 kDa; complex III, subunit core 2-"CIII-core2"-47 kDa; complex IV, subunit II-"CIV-II"-24 kDa; Complex V, ATP synthase subunit alpha-"CV-α"-53 kDa; COX, cytochrome C oxidase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MTSSB, mitochondrial single strand DNA binding protein; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; TFAM, mitochondrial transcription factor.

*Significant difference between leptin-treated and untreated cells (Student's *t*-test; $P \leq 0.05$).

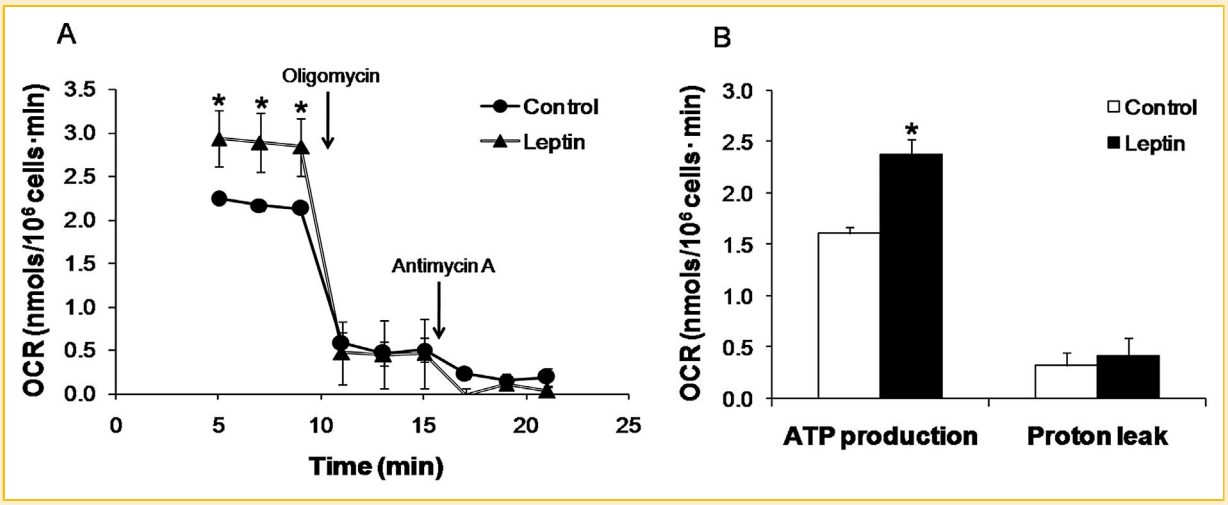


Fig. 2. Oxygen consumption rate. (A) Leptin-treated cells exhibited a higher basal respiration rate than control cells. Respiration rate dropped to similar levels in control and leptin-treated cells after ATP synthase inhibition (oligomycin) and no differences were found after inhibition of electron transport chain (antimycin A). (B) Histograms show ATP synthase- or proton leak-dependent oxygen consumption. Leptin increased the oxygen consumed for ATP production in basal conditions whereas the oxygen consumed for proton leak was unaltered. Data represent means ± SEM (n = 3). *Significant difference between leptin-treated and untreated cells (Student's *t*-test; $P < 0.05$). 2-DG, 2-deoxyglucose; OCR, oxygen consumption rate.

[Soga, 2013; Wu and Zhao, 2013]. To test whether leptin may shift the source of ATP production, we measured ATP levels after the addition of 2-DG and oligomycin (glycolysis and mitochondrial ATP synthase inhibitors, respectively). As shown in Figure 1B, after 24 h of leptin exposure, cellular ATP levels were more reliant on mitochondria in leptin-treated cells compared to the more glycolysis-dependent metabolism of control cells. In accordance, the rate of glycolysis measured by the release of lactate was decreased after 24 h of leptin treatment in MCF-7 cells (Fig. 1C). Nevertheless, as shown in the figure, the ability to activate glycolysis after mitochondrial respiration inhibition with oligomycin was preserved in both leptin and control cells.

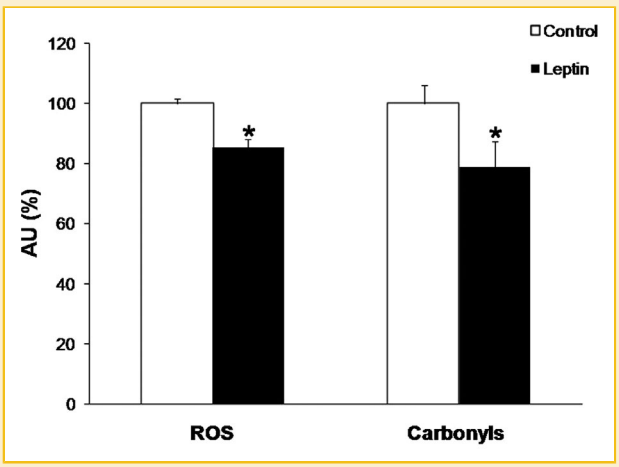


Fig. 3. Oxidative stress status. Histograms show the ROS production and carbonyls levels in control and leptin-treated cells. MCF-7 cells decreased H_2O_2 production and total protein carbonyl levels, thus ameliorating oxidative stress status. Control values were set at 100, data are expressed in arbitrary units and represent means ± SEM (n = 6). *Significant difference between leptin-treated and untreated cells (Student's *t*-test; $P < 0.05$). AU, arbitrary units; ROS, reactive oxygen species.

To explore how leptin increases mitochondrial metabolism we measured the expression of key markers of mitochondrial biogenesis and function. As shown in Table 2, leptin increased the expression of *PGC-1α* ($P < 0.05$), the master regulator of mitochondrial biogenesis [Jager et al., 2007; Rohas et al., 2007]. In agreement, it has been reported an enhanced expression of PGC-1α with leptin in adipocytes [Luo et al., 2008]. One of the roles of PGC-1α is to promote the expression of TFAM, the transcription factor involved in the expression of mitochondrial genome codified genes. We did not find statistical differences in mRNA expression of *TFAM*, but we observed higher protein levels by western blot (Table 2). This apparent discrepancy between mRNA and protein TFAM levels may be explained by regulation at translational level. In fact, Jiang et al. [2013] have recently found that *TFAM* expression may be regulated by miRNAs in glioma cells. These authors showed that TFAM protein levels were increased by down-regulation of miRNA-23b, which represses *TFAM* mRNA translation. *MTSSB*, another important factor necessary for mtDNA replication and thus for novel mitochondria formation, was also increased by leptin (Table 2). Increased expression of these biogenesis-related markers depicts a scenario of new mitochondria formation, according to the accelerated cell proliferation induced by leptin. Surprisingly, we found no differences in OXPHOS protein levels or in maximal ATP synthase or COX activities after leptin treatment (Table 2). These puzzling findings could be explained if leptin increases mitochondrial functionality rather than mitochondrial mass. To test this assumption, we analyzed cellular oxygen consumption in live cells. Oxygen consumed by mitochondria can be divided into: (i) oxygen

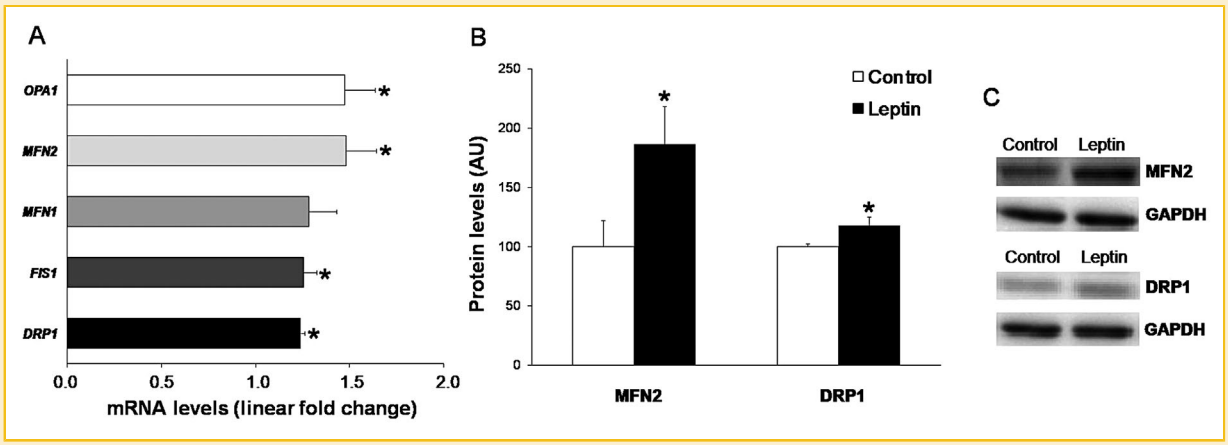


Fig. 4. Expression of mitochondrial fusion and fission related-genes and protein levels. A) Six hours of leptin-treatment elevated mRNA levels of fusion (*OPA1*, *MNF1*, *MFN2*) and fission (*FIS1*, *DRP1*) related-genes. Data are expressed as linear fold change (Leptin vs Control) and positive values indicate up-regulation. B) Histograms show an increase in MFN2 and DRP1 protein levels in leptin-treated cells for 48 h. Control values were set at 100, data are expressed in arbitrary units and represent means ± SEM (n = 6). C) Immunoblots showing representative bands of MFN2, DRP1, and GADPH as housekeeping. * Significant difference between leptin-treated and untreated cells (Student's *t*-test; $P \leq 0.05$). DRP1, dynamin-related protein-1; FIS1, mitochondrial fission protein 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MFN1, mitofusin-1; MFN2, mitofusin-2; OPA1, optic atrophy-1.

consumed for ATP production and (ii) oxygen consumed for proton leak [Brand and Nicholls, 2011]. These two components can be estimated by measuring the OCR in the presence of specific inhibitors. As shown in Figure 2A and B, basal OCR was significantly higher in leptin-treated cells compared to untreated ones. After oligomycin addition, OCR dropped to similar levels in both leptin and control cells. The difference between basal- and oligomycin-OCR is the amount of oxygen that is consumed for ATP production [Brand and Nicholls, 2011]. As shown, leptin increases basal oxygen consumption in an ATP synthase-dependent manner. On the other hand, in order to assess the mitochondrial proton leak, we added antimycin A to entirely inhibit mitochondrial oxygen consumption. The difference between oligomycin- and antimycin A-OCR allows the calculation of the oxygen consumed by proton leak. We did not find differences in proton leak between leptin and control cells. Altogether, OCR analysis suggests that leptin activates state 3 respiration, increasing ATP production, without alteration of uncoupling.

An improvement in mitochondrial function may be revealed not only by increased bioenergetic efficiency but also by a low

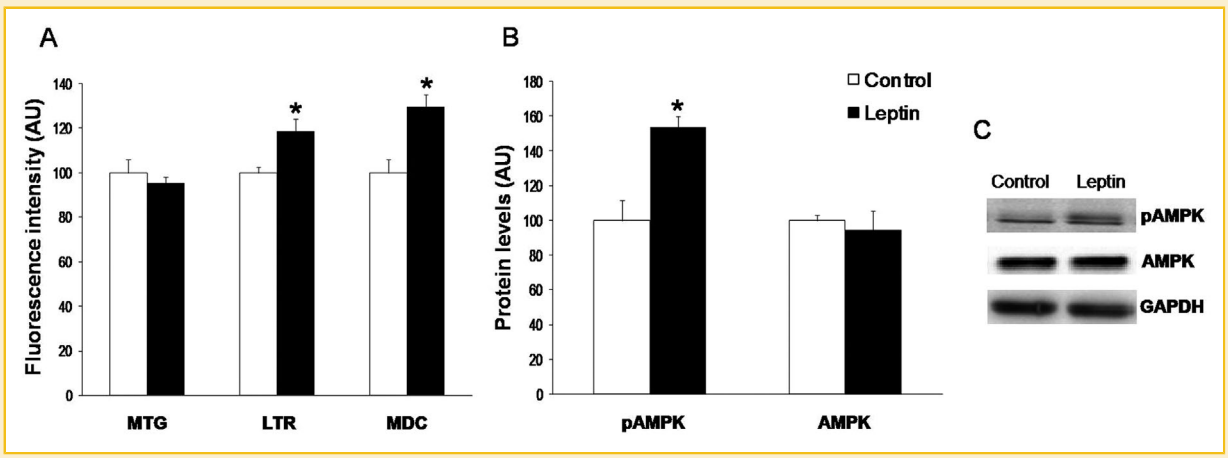


Fig. 5. Mitochondrial and lysosomal mass and autophagy assay. A) Cells treated with leptin showed an increase in the uptake of the fluorescent probe lysotracker red (LTR) and the specific autophagosome probe monodansylcadaverine (MDC). No differences were observed in the uptake of the mitochondrial specific probe (MTG). B) Histograms showing an increase in the phosphorylation of the master regulator of autophagy AMPK. Control values were set at 100, data are expressed in arbitrary units and represent means ± SEM (n = 6). C) Immunoblots showing representative bands of pAMPK, AMPK, and GADPH as housekeeping. * Significant difference between leptin-treated and untreated cells (Student's *t*-test; $P < 0.05$). AMPK, 5′ AMP-activated protein kinase; AU, arbitrary units; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MDC, monodansylcadaverine; MTG, mitotracker green; LTR, lysotracker red; pAMPK, phosphorylated 5′ AMP-activated protein kinase.

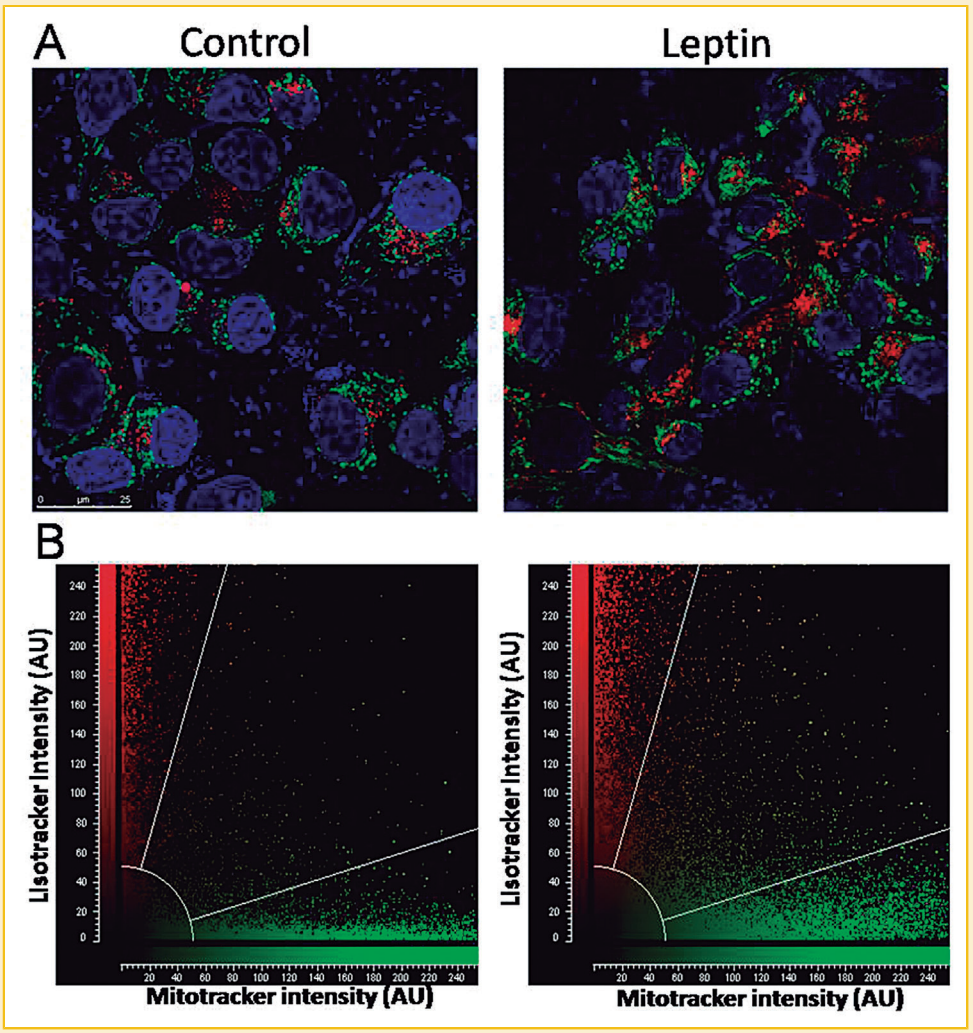


Fig. 6. Confocal microscopy imaging. A) Cells were co-stained with MTG for mitochondria (green), LTR for lysosomes (red) and Hoechst 33342 for nuclei (blue). B) Cytofluorograms show that cells treated with leptin present an increased colocalization between lysosomes and mitochondria. AU, arbitrary units.

production of ROS, the toxic byproduct of oxygen metabolism. To evaluate mitochondria from this point of view, oxidative stress parameters such as H_2O_2 production and carbonyl protein levels were determined. Figure 3 shows a statistically significant decrease in the levels of both parameters after 24 h of leptin exposure (−15% and −20%, respectively). The amelioration of oxidative damage along with the higher ATP production suggests that leptin improves mitochondrial respiratory chain function.

At this point, the question about how leptin improves mitochondrial function on MCF-7 breast cancer cells may arise. Mitochondria are highly dynamic organelles and undergo constant fusion and fission, processes essential for maintaining physiological functions of cells and help maximize the capacity for oxidative phosphorylation under stressful conditions [Grandemange et al., 2009; Youle and van der Bliek, 2012]. To determine whether leptin affects mitochondrial dynamics, mRNA and levels of proteins involved in fusion (OPA1, MFN1, and MFN2) and fission (FIS1 and DRP1) were examined. Figure 4A shows that the expression of *OPA1, MFN2, FIS1* and *DRP1* genes was significantly increased by leptin in MCF-7 cells. Higher levels of the proteins MFN2 and DRP1 were confirmed by western blot (Fig. 4B). Mitochondrial fusion helps to mitigate stress by mixing and complementing the contents of partially damaged mitochondria, whereas fission is part of the process of mitochondrial biogenesis but also contributes to quality control by enabling the removal of damaged mitochondria by mitophagy [Novak, 2012; Youle and van der Bliek, 2012]. Accelerated fusion–fission cycles, in balance with increased biogenesis and mitophagy, allow cells to purge damaged mitochondria by its excision from the network and their autophagy by lysosomes [Chan, 2012; Youle and van der Bliek, 2012]. In this regard, a net increase of lysosomes (+19%) and autophagosomes (+30%) without net changes in mitochondrial mass were observed in cells exposed to leptin using the specific probes LTR, MDC, and MTG, respectively (Fig. 5A). Furthermore, leptin induced the phosphorylation of AMPK, considered the master regulator of autophagy (Fig. 5B) [Hoyer-Hansen et al., 2007; Kim et al., 2011]. Confocal microscopy analysis

of co-stained cells with LTR and MTG showed a greater colocalization between mitochondria and lysosomes after leptin treatment, which points towards an increased mitophagy induced by leptin (Fig. 6). Thus, accelerated fusion–fission cycles in balance with increased biogenesis and mitophagy may be a mechanism by which leptin allows cells to purge a greater number of damaged mitochondria.

Leptin is known to modulate cell metabolism through central nervous system and directly in leptin-sensitive-tissues [Anubhuti and Arora, 2008]. Previous work using ob/ob mice found that hepatic basal metabolic rate was decreased with leptin as a result of a reduction in mitochondrial volume and mitochondrial respiratory chain components [Singh et al., 2009]. Although these findings in liver are opposite to our findings in breast cancer cells, both studies evidence the ability of leptin to modulate mitochondrial metabolism in different tissues. Previous work by Park et al. [2010] using a breast cancer model of db/db mice deficient in Ob-R in peripheral tissues found strong evidence that leptin signaling supports the Warburg effect by suppressing mitochondrial respiration in vivo. Here, in the widely used MCF-7 breast cancer cells, we have found higher mitochondrial oxygen consumption without changes in mitochondrial volume density or respiratory chain proteins. In agreement with our study, other authors have reported that leptin increases mitochondrial biogenesis through PGC-1α activation in muscle [Jager et al., 2007; Rohas et al., 2007]. Regarding to the molecular mechanisms involved in leptin action, previous studies have shown that leptin selectively stimulates phosphorylation and activation of AMPK in skeletal muscle which in turn stimulates fatty-acid oxidation [Minokoshi et al., 2002]. It is well known that activation of AMPK results in mitochondrial biogenesis in skeletal muscle [Bergeron et al., 2001; Zong et al., 2002] and reduction of intracellular ROS levels [Kukidome et al., 2006; Ouedraogo et al., 2006]. Thus, in contrast to the study of Park et al. [2010] our results support a role for leptin increasing oxidative metabolism in breast cancer cells, similar as occurs in muscle cells [Minokoshi et al., 2002]. A common candidate mechanism of the action of leptin in mitochondria of breast cancer and muscle cells could be the activation of the energy sensor AMPK. This enzymatic complex acts as a signaling hub orchestrating mitochondrial biogenesis, metabolism, and autophagy, allowing cells to set up the mitochondrial machinery to face an energetic challenge. Future work addressing leptin effects in breast cancer compared to normal breast cell lines would also be of interest to determine specific features of leptin role on cancerous metabolism. On the other hand, further studies using a wider panel of cell lines and other animal models such as the ob/ob mice will shed more light on the complex relationship between leptin and breast cancer metabolism.

CONCLUSIONS

To sum up, our results suggest a role for leptin in mitochondrial biogenesis and dynamics with an amelioration of oxidative stress and higher mitochondrial ATP production. Changes in mitochondria quality control or metabolic reprogramming may be underlying leptin action, which probably confers benefits for the growth and survival of breast cancer cells.

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