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Metabolism Clinical and Experimental 55 (2006) 1067–1075

Metabolism
Clinical and Experimental

www.elsevier.com/locate/metabol

Distinct effects of short- and long-term leptin treatment on glucose and fatty acid uptake and metabolism in HL-1 cardiomyocytes

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Received 12 January 2006; accepted 8 March 2006

Abstract

Alterations in cardiac glucose and fatty acid metabolism are possible contributors to the pathogenesis of heart failure in obesity. Here we examined the effect of leptin, the product of the obese (*ob*) gene, on metabolism in murine cardiomyocytes. Neither short-term (1 hour) nor long-term (24 hours) treatment with leptin (60 nmol/L) altered basal or insulin-stimulated glucose uptake and oxidation, glycogen synthesis, insulin receptor substrate 1 tyrosine, Akt, or glycogen synthase kinase 3β phosphorylation. Extracellular lactate levels were also unaffected by leptin. However, leptin increased basal and insulin-stimulated palmitate uptake at both short and long exposure times and this corresponded with increased cell surface CD36 levels and elevated fatty acid transport protein 1 (FATP1) and CD36 protein content. Whereas short-term leptin treatment increased fatty acid oxidation, there was a decrease in oxidation after 24 hours. The former corresponded with increased acetyl coenzyme A carboxylase phosphorylation and the latter with increased expression of this enzyme. The discrepancy between uptake and oxidation of fatty acids led to a transient decrease in intracellular lipid content with lipid accumulation ensuing after 24 hours. In summary, we demonstrate that leptin did not alter glucose uptake or metabolism in murine cardiomyocytes. However, fatty acid uptake increased while oxidation decreased over time leading to intracellular lipid accumulation, which may lead to lipotoxic damage in heart failure.

1. Introduction

Given the increasing prevalence of obesity [1] and its association with the development of heart failure [2], understanding the mechanisms underlying this association is imperative. There is no single direct cause of heart failure, its manifestation instead being the end result of a variety of cardiovascular abnormalities. When cardiac tissue is damaged in any way, which compromises the function of the heart, compensatory responses are initiated, which at first help to maintain blood flow but ultimately lead to extra strain on the failing heart. The series of adaptive changes within the myocardium are collectively referred to as remodeling and occur primarily in the left ventricle [3,4]. Broadly speaking, the changes that occur include those affecting size or number of myocytes, and those altering the composition and structure of the extracellular matrix and altered energy metabolism [4,5].

Adipose tissue, once considered simply a lipid storage depot, is now known to be a dynamic endocrine organ [6]. Because progression of heart failure is now commonly believed to result due to a complex interplay of detrimental effects mediated by a variety of hormones on the myocardium, this study will focus on the adipose-derived hormone ("adipokine") leptin, the product of the obese (*ob*) gene [7], which has been proposed to play a role in the pathophysiology of many obesity-related disorders. Elevated plasma leptin levels are found in patients with congestive heart failure [8], and fasting plasma leptin concentrations in hypertensive men were significantly associated with myocardial wall thickness, independent of hypertension [9,10]. The ability of leptin to attenuate cardiac muscle contraction has been proposed to explain the decrease in diastolic compliance and prolonged relaxation observed in obese individuals [11]. Moreover, both the ability of leptin to regulate fatty acid oxidation and thus triglyceride content in isolated rat hearts or to alter adenylate cyclase function in a cultured cardiac muscle cell line have been suggested to represent a potential mechanism leading to altered cardiac function in obese individuals [12,13].

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0026-0495/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.metabol.2006.03.020

Obesity and the typically associated diabetes are correlated with profound alterations in cardiac energy metabolism, which can lead to compromised ventricular performance [14,15]. In obesity and diabetes, excessive fatty acid oxidation and decreased glucose uptake and oxidation occur, leading to the heart being more reliant on fatty acids as a source of energy. It has been suggested that enhancing glucose utilization may be beneficial in preventing cardiomyopathy in obesity and diabetes [16,17]. In addition, perturbations in fatty acid metabolism leading to accumulation of lipotoxic products can mediate many detrimental effects on the heart, including apoptosis, impairing contractile function, inducing or exacerbating arrhythmias, and changes in cell signaling and membrane function [18,19]. Here we investigated the effect of leptin at different periods on cardiomyocyte glucose and fatty acid uptake and oxidation and associated regulatory proteins.

2. Experimental procedures

2.1. Materials

Recombinant murine leptin was obtained from Calbiochem (San Diego, CA) and human insulin (Humulin) from Eli Lilly (Toronto, Canada). 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside was purchased from Toronto Research Chemicals (Toronto, ON, Canada). [1-^{14}C]Palmitate, D-[U-^{14}C]glucose, and 2-deoxy-D-[^{3}H]glucose were from Amersham (Quebec, Canada). 4,4-Difluoro-5,7-dimethyl-4-boro-3a,4a-diaza-s-indacene-3-3hexadecanoic acid (BODIPY)-conjugated palmitate was purchased from Molecular Probes (Eugene, OR). Claycomb medium was from JRH Biosciences (Lenexa, KS). Fatty acid transport protein 1 (FATP1), FATP4, FAT/CD36, adenosine monophosphate–activated protein kinase α1 (AMPKα1), and AMPKα2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-AMPK (Thr-172), acetyl coenzyme A (CoA) carboxylase (ACC), phospho-ACC (Ser-79), phospho-Akt (Ser-473) and (Thr-308), phospho-glycogen synthase kinase-3β (GSK-3β, Ser-9) primary antibodies, and horseradish peroxidase (HRP)–conjugated antirabbit secondary antibody were purchased from Cell Signaling Technology (Beverly, MA). Phospho-insulin receptor substrate 1 (IRS-1) Tyr-612 antibodies were obtained from Biosource (Camarillo, CA). Goat antimouse HRP-conjugated secondary antibody was from Biorad (Hercules, CA) and sheep antigoat HRP-conjugated secondary antibody was from Calbiochem (San Diego, CA). Enhanced chemiluminescence reagent was purchased from PerkinElmer Life Sciences (Boston, MA). Oil red O and triethyl phosphate were from Fluka Chemie (Buchs, Switzerland). All other reagents were of the highest grade available.

2.2. Cell culture

HL-1 cells were maintained as previously described [20] using Claycomb medium supplemented with 10% (vol/vol) fetal bovine serum, 4 mmol/L L-glutamine, 10 μmol/L noradrenaline (norepinephrine; Sigma, St Louis, MO), and 1% (vol/vol) penicillin-streptomycin. Cells were grown at 37°C, in an atmosphere of 5% CO_2. During continued growth the medium was changed routinely every 48 hours.

2.3. Measurement of 2-deoxy-D-[^{3}H]glucose uptake

Cells grown in a 24-well plate were treated with leptin (60 nmol/L, 1 or 24 hours) and/or insulin (100 nmol/L, 20 minutes). Cells were serum starved for 5 hours, then glucose transport was assayed for 5 minutes at room temperature as previously described [21]. The incubation medium was aspirated, the cells were washed with ice-cold saline, and 200 μL of KOH (1 mol/L) was added to each well. Aliquots of cell lysates were transferred to scintillation vials for radioactivity counting and the remainder used for protein assay. Nonspecific uptake was determined in the presence of cytochalasin B (10 μmol/L) and was subtracted from all values. Results are calculated as picomole of glucose uptake per minute per milligram of protein and control values assigned an arbitrary value of 1.

2.4. Measurement of fatty acid uptake

Fatty acid uptake was determined as described previously [22] with minor modifications. Briefly, cells were grown on coverslips in 12-well plates and, after treatment with or without leptin (60 nmol/L, 1 or 24 hours), cells were serum starved for 5 hours in the continued presence of leptin and incubated with insulin (100 nmol/L, 20 minutes). Medium was aspirated, and the cells were washed twice with phosphate-buffered saline (PBS) containing fatty acid–free albumin. Cells were then incubated with BODIPY-conjugated palmitate (1 μmol/L) for 2 minutes at 37°C, and the coverslips washed 3 times with cold PBS and mounted on clean glass slides using Dako antifade solution (Dako Corp, Carpinteria, CA). Nonspecific uptake is determined via competition with 5 mmol/L palmitate. For confocal microscopy analysis, BODIPY-conjugated fatty acids were excited at 488 nm with an Olympus (Melville, NY) FluoView 300 multiline argon laser. Fluorescent images were obtained using FluoView software and fluorescence intensity quantitated by ImageJ software.

2.5. Measurement of glycogen synthesis

Glycogen synthesis was measured by the incorporation of D-[U-^{14}C]glucose to glycogen as we described previously [23] with some modifications. Briefly, cells were grown in 6-well plates and preincubated with or without leptin (60 nmol/L, 1 or 24 hours) and deprived of serum for 3 to 5 hours before incubation with 0.15 μCi/mL D-[U-^{14}C]glucose in the presence or absence of insulin (100 nmol/L) for 2 hours. The cells were washed 3 times with cold PBS and lysed in 1 N KOH. For measurement of insulin-stimulated incorporation of glucose into glycogen, cell lysates were used for overnight glycogen precipitation with ethanol. Precipitated glycogen

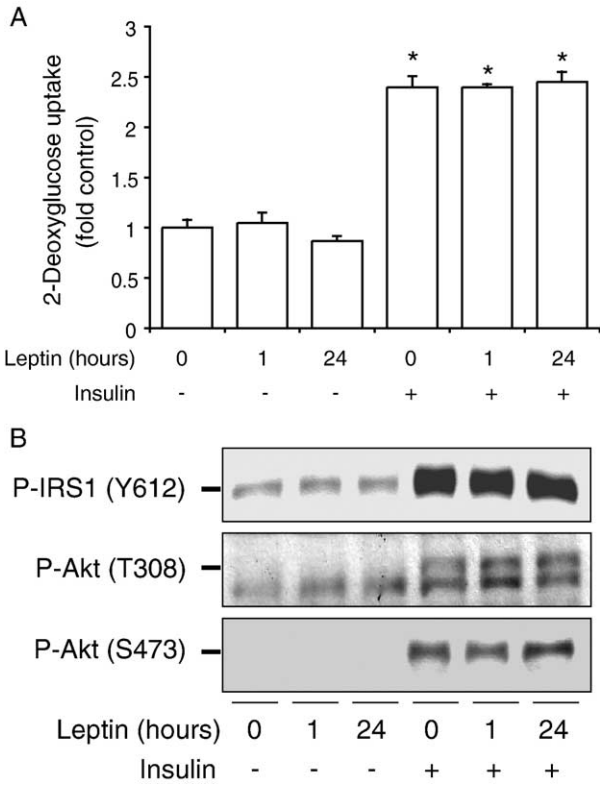

Fig. 1. Regulation of glucose uptake and associated signaling pathways. We examined the effect of pretreating HL-1 cells with leptin (60 nmol/L) for 1 or 24 hours on basal and insulin-stimulated (100 nmol/L, 20 minutes) glucose uptake as shown in A. The results presented are mean ± SEM (n = 6). *P < .05, significant with respect to control. The effect of similar leptin pretreatments on basal and insulin-stimulated (100 nmol/L, 10 minutes) phosphorylation of IRS-1 on Y612 and Akt on T308 and S473 is shown in B. A representative Western blot from 4 experiments is shown.

was then dissolved in water and transferred to scintillation vials for radioactivity counting.

2.6. Analysis of lactate production

Lactate content was determined by the lactate oxidase method using a lactate assay kit (Sigma). Cells were incubated with or without leptin (60 nmol/L, 1 or 24 hours), followed by serum starving for 5 hours in the continued presence of leptin. Cells were then incubated with insulin (100 nmol/L, 2 hours), after which the media was collected and used for analysis.

2.7. Analysis of cell surface CD36 content

We used an antibody-coupled colorimetric assay as we described previously to measure cell surface CD36 content [22]. Briefly, cells were grown in 12-well plates with or without leptin (60 nmol/L, 1 or 24 hours), followed by 5 hours of serum deprivation in the continued presence of leptin and incubated plus or minus insulin (100 nmol/L, 20 minutes). Subsequently, cells were quickly washed in ice-cold PBS and incubated with anti-CD36 polyclonal antibody (H300, Santa Cruz Biotechnology, 1:200 dilution) for 60 minutes at 4°C. Cells were washed and fixed in 3% paraformaldehyde for 3 minutes on ice. The fixative was then neutralized by incubation in 10 mmol/L glycine in ice-cold PBS for 10 minutes. Cells were blocked in 10% goat serum for 10 minutes and then incubated with HRP-conjugated goat antirabbit immunoglobulin G (1:1000 dilution, 4°C) for 60 minutes. Cells were washed 5 times with ice-cold PBS and incubated for 30 minutes at room temperature with 1 mL of OPD reagent (0.4 mg/mL *O*-phenylenediamine dihydrochloride and 0.4 mg/mL urea hydrogen peroxide in 0.05 mol/L phosphate citrate buffer) per well. The reaction was stopped by adding 0.25 mL of HCl (3 mol/L). The supernatant was collected, and the absorbance was measured at 492 nm. Absorbance associated with nonspecific binding (primary antibody omitted) was used as a blank.

2.8. Measurement of intracellular lipid accumulation by oil red O staining

Determination of lipid content was carried out as described by us previously [22] with minor modifications. Cells were grown on coverslips in 12-well plates and preincubated with or without leptin (60 nmol/L, 1 or 24 hours) followed by serum starving for 3 hours in the continued presence of leptin. Cells were then treated with insulin (100 nmol/L, 3 hours) where indicated. After this period, cells were fixed in 3.7% formaldehyde for 60 minutes, and excess of formaldehyde was removed by 3 rinses in deionized water for 30 seconds. Subsequently, oil red O staining was carried out as described previously [22]. Images were obtained on a laser scanning confocal microscope (Olympus FluoView 300, 60× objective) using HeNe laser (Texas red excitation filter at 543 nm). The intensity of lipid droplets/cytoplasmic area was then analyzed quantitatively by using ImageJ software.

2.9. Measurement of glucose and fatty acid oxidation

Glucose and fatty acid oxidation was measured by the production of $^{14}CO_2$ from D-[U-^{14}C]glucose and [1-^{14}C]palmitate, respectively, as previously described [22,23] with a few modifications. Briefly, cells were cultivated in 60 × 15-mm Petri dishes with or without leptin (60 nmol/L, 1 or 24 hours). After serum deprivation for 3 to 5 hours in the continued presence of leptin, cells were incubated for 2 hours with insulin (100 nmol/L), where indicated, in medium containing 0.15 μCi/mL D-[U-^{14}C]glucose or D-[1-^{14}C]palmitate. Each Petri dish was sealed with parafilm that had a piece of Whatman paper taped facing the inside of the Petri dish. After 2 hours of incubation the Whatman paper was wet with 100 μL of phenylethylamine-methanol (1:1) to trap carbon dioxide produced during the incubation period, then 200 μL of H_2SO_4 (4 mol/L) was then added. After incubation for 1 hour at 37°C, the pieces of Whatman paper were removed and transferred to scintillation vials for radioactivity counting.

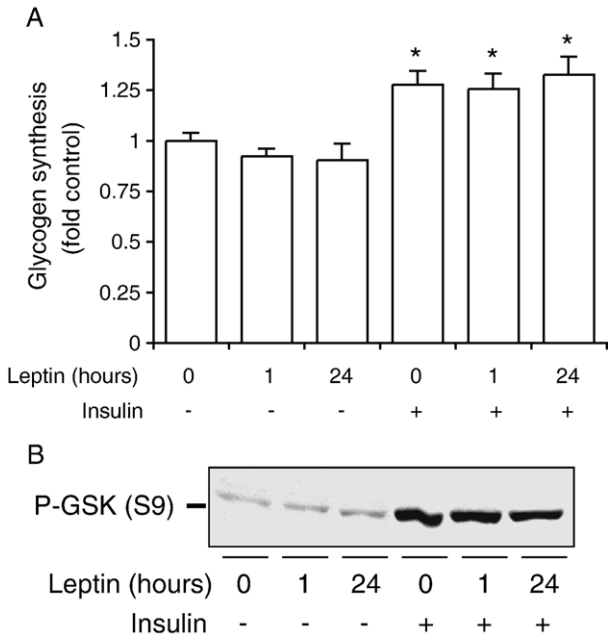

Fig. 2. Regulation of glycogen synthesis and GSK-3β phosphorylation. Here, we pretreated HL-1 cells with leptin (60 nmol/L) for 1 or 24 hours and then examined the effect on basal and insulin-stimulated (100 nmol/L, 2 hours) glycogen synthesis. The results shown in A represent mean ± SEM (n = 4). *P < .05, significant with respect to control. The effect of similar leptin pretreatments on basal and insulin-stimulated (100 nmol/L, 10 minutes) phosphorylation of GSK-3β on S9 is shown in B. A representative Western blot from 3 experiments is shown.

2.10. Preparation of cell lysates and immunoblotting

Lysates were prepared essentially as previously described [20]. Cells were grown in 6-well plates and treated with or without leptin (60 nmol/L, 1 or 24 hours), followed by 3 to 5 hours of serum deprivation in the continued presence of leptin and incubated plus or minus insulin (100 nmol/L) for the times indicated in figure legends. Plates were washed 3 times with ice-cold PBS, then 200 μL of Laemmli sample buffer containing protease and phosphatase inhibitors (0.5 mmol/L Na_3VO_4, 10 mmol/L NaF, 1 μmol/L leupeptin, 1 μmol/L pepstatin, 1 μmol/L okadaic acid, 0.2 mmol/L phenylmethylsulfonyl fluoride) was added. The lysates were boiled at 65°C for 5 minutes and were syringed 5 times then centrifuged at 12 000 rpm for 1 minute at 4°C, and the supernatant was used for further analysis. An aliquot of the cell lysate was used to determine the protein concentration in each sample. Before loading onto sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis gels, the samples were diluted 1:1 (vol/vol) with 2× Laemmli sample buffer (62.5 mmol/L Tris-HCl [pH 6.8], 2% [wt/vol] SDS, 50 mmol/L dithiothreitol (DTT), 0.01% [wt/vol] bromophenol blue). Aliquots of cell lysates containing 20 μg of protein were then subjected to SDS–polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes. The expression level of AMPKα-1, AMPKα-2, ACC, FATP1, FATP4, and CD36 was determined using specific antibodies. Phosphorylation level of insulin receptor substrate (Tyr-612), Akt (Thr-308 and Ser-473), GSK-3β (Ser-9), AMPK (Thr-172), and ACC (Ser-79) was detected using phospho-specific antibodies. Primary antibody detection was performed using HRP-conjugated appropriate secondary antibody, visualized using enhanced chemiluminescence, and quantified using the Scion Image program.

2.11. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide assay

Cell viability was determined using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich). Briefly, cells were seeded at a density of 1×10^6 cells/mL in 96-well plates and incubated in the presence or absence of leptin (60 nmol/L) for up to 24 hours. MTT was then added, and the ability of cells to reduce this substrate to the blue formazan product was determined colorimetrically (550 nm) as an indicator of cell viability.

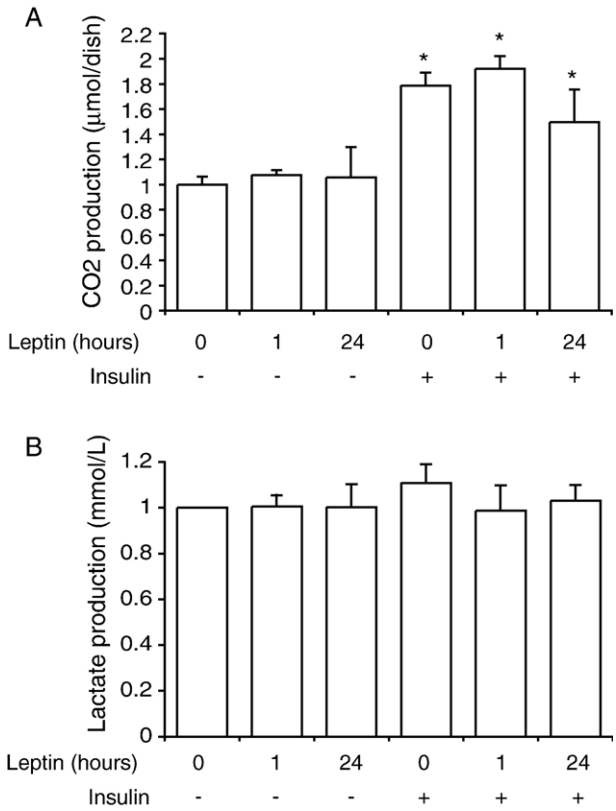

Fig. 3. Regulation of glucose metabolism via oxidation and lactate production. We examined the effect of pretreating HL-1 cells with leptin (60 nmol/L) for 1 or 24 hours on basal and insulin-stimulated (100 nmol/L, 2 hours) glucose oxidation (A) and lactate production (B). In each case the results presented are mean ± SEM (n = 3). *P < .05, significant with respect to control.

2.12. Statistical analysis

Data are expressed as means ± SEM. Statistical analysis was undertaken using paired Student *t* test. Differences between groups were considered statistically significant when $P < .05$.

3. Results

We first examined the effect of leptin on [^{3}H]2-deoxy-glucose uptake in HL-1 cardiomyocytes and found that basal uptake was unaffected by leptin preincubation for 1 or 24 hours (Fig. 1A). The dose of leptin we have chosen to use in this study was based on both preliminary experiments and our published observations [20,24]. Furthermore, no difference in cell viability, measured by MTT assay, was observed after up to 24 hours of leptin treatment (data not shown). Insulin has been previously shown to stimulate glucose uptake in cardiomyocytes and, here, we observed a significant increase of almost 2.5-fold in response to insulin, which was not altered by prior incubation with leptin for 1 or 24 hours (Fig. 1A). In keeping with the fact that leptin had no effect on glucose uptake, we also observed no effect of leptin on basal or insulin-stimulated phosphorylation of signaling proteins that play a well-characterized role in glucose uptake, namely, IRS-1 (Y612), Akt (T308), and Akt (S473) (Fig. 1B). We also investigated the effect of leptin on metabolism of glucose and found that basal and insulin-stimulated glycogen synthesis and GSK-3β (S9) phosphorylation were unaffected by leptin pretreatment for 1 or 24 hours (Fig. 2). Both basal and insulin-stimulated glucose oxidation, measured by carbon dioxide production, was also unaffected by leptin (Fig. 3A). There was no change in the lactate content of media from cells treated with insulin or leptin (Fig. 3B).

Fatty acid uptake was measured using fluorescently labeled palmitate, and we found a significant increase in response to leptin at both 1 and 24 hours (Fig. 4A and B). The ability of insulin to stimulate fatty acid uptake was also potentiated by prior incubation of cells with leptin for 24 hours (Fig. 4A and B). The increased fatty acid uptake elicited by insulin and leptin at both 1 and 24 hours correlated with increased cell surface content of CD36 (Fig. 4C). We also examined the effect of leptin on expression of 3 fatty acid transporters found in these cells and found that leptin increased the level of FATP1 and CD36 protein while having no effect on the total content of FATP4 (Fig. 4D). Oxidation of palmitate was increased after 1 hour of leptin treatment, but decreased after prolonged (24 hours) exposure of cells to

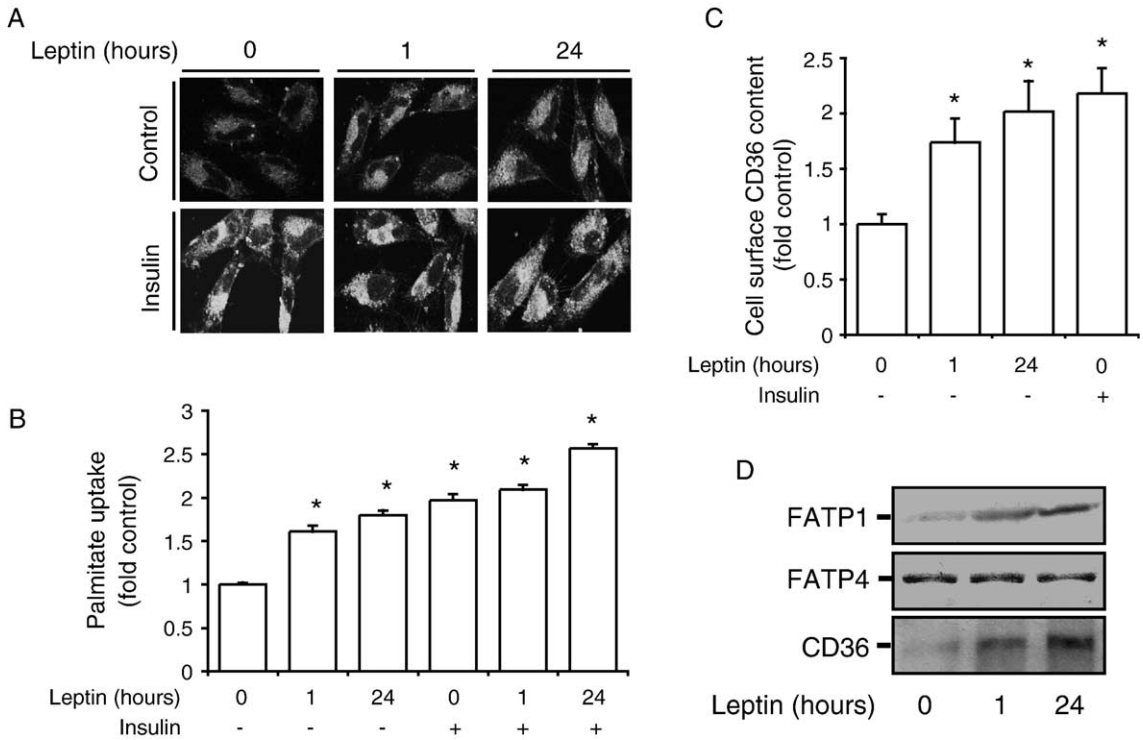

Fig. 4. Regulation of fatty acid uptake and fatty acid transporter expression and localization. We examined the effect of pretreating HL-1 cells with leptin (60 nmol/L) for 1 or 24 hours on basal and insulin-stimulated (100 nmol/L, 20 minutes) uptake of fluorescently labeled palmitate. Shown in A are representative immunofluorescent images, and the results presented in B show quantitative analysis, mean ± SEM of 4 experiments (with total analysis of >50 cells from several distinct fields of view). *$P < .05$, significant with respect to control. We then analyzed the effect of leptin (60 nmol/L, 1 or 24 hours) on translocation of CD36 to the cell surface, with insulin (100 nmol/L, 20 minutes) shown as positive control. Results shown in C represent mean ± SEM (n = 3). *$P < .05$, significant with respect to control. A representative Western blot from 4 experiments examining the effect of leptin (60 nmol/L, 1 or 24 hours) on expression of FATP1, FATP4, and CD36 is shown in D.

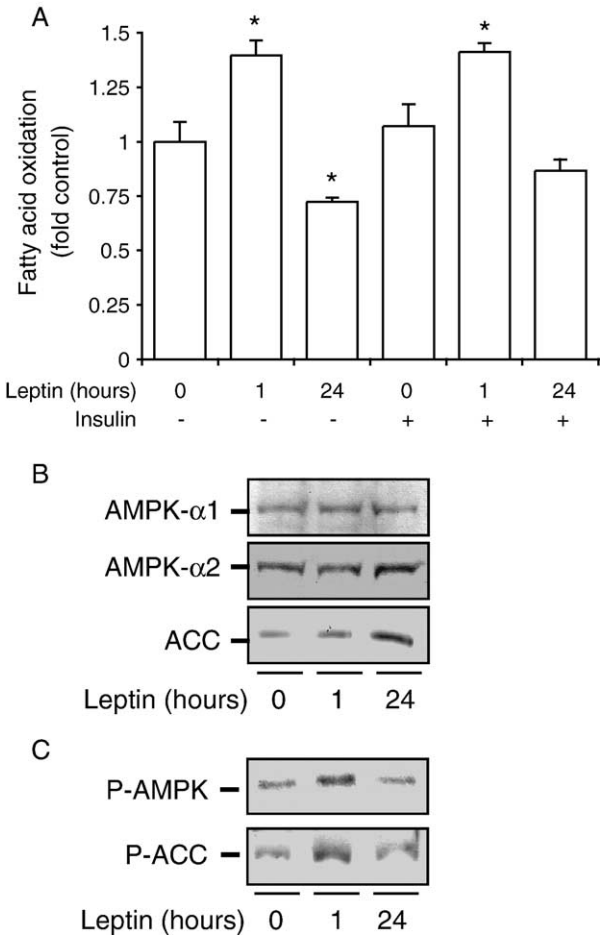

Fig. 5. Regulation of fatty acid oxidation and AMPK and ACC expression and phosphorylation. We examined the effect of pretreating HL-1 cells with leptin (60 nmol/L) for 1 or 24 hours on basal and insulin-stimulated (100 nmol/L, 2 hours) fatty acid oxidation, and data shown in A represent mean ± SEM (n = 4) *$P < .05$, significant with respect to control. Also shown is a representative Western blot (n ≥ 3) documenting the effect of leptin (60 nmol/L, 1 or 24 hours) pretreatment on expression of AMPK isoforms and ACC (B) and phosphorylation of AMPK and ACC (C).

leptin, whereas there was no effect of insulin on oxidation of fatty acid in these cells (Fig. 5A). These effects on fatty acid oxidation correlated with increased AMPK and ACC phosphorylation after 1 hour (Fig. 5C). After 24 hours, phosphorylation of AMPK and ACC and AMPK expression were similar to control; however, expression of ACC was increased (Fig. 5B and C). A slight decrease in intracellular lipid accumulation was observed after 1 hour of leptin treatment, whereas the discrepancy between fatty acid uptake and oxidation after 24 hours of leptin treatment caused a significant increase in lipid accumulation (Fig. 6).

4. Discussion

Fatty acid oxidation normally provides ~70% of adenosine triphosphate (ATP) necessary for the heart to maintain contractile function [18]. Circulating fatty acids, chylomicron-triacylglycerol, and very low-density lipoprotein triacylglycerol (TAG) as well as myocardial TAG stores are additional critical fuel sources, yet the remaining 30% of myocardial ATP is largely derived from glucose and lactate. Energy production from fatty acids requires oxygen consumption, whereas carbohydrate-derived ATP is produced by both glycolysis (oxygen independent) and glucose oxidation. Thus, although glucose represents a small component of total myocardial energy source, it is the most efficient means of energy production, particularly in times of ischemia/hypoxia. Specifically, the equivalent amount of ATP production from fatty acid oxidation consumes 10% more oxygen [14]. In general, glucose transport, glycolysis, and glucose oxidation in cardiomyocytes decrease and fatty acid uptake and oxidation are increased in obesity and diabetes [14,15,25,26]. In cases such as this and in ischemic heart failure it is beneficial to promote glucose metabolism, which has an oxygen-independent component (glycolysis) [27,28]. However, in cases of idiopathic heart failure a decreased fatty acid metabolism is typically observed; thus, under such circumstances it may be beneficial to promote fatty acid metabolism [27]. Increased myocardial oxygen consumption was observed in hearts from leptin-deficient (*ob/ob*) mice, indicating that these obese animals were metabolically inefficient [29]. Overall, defining the exact

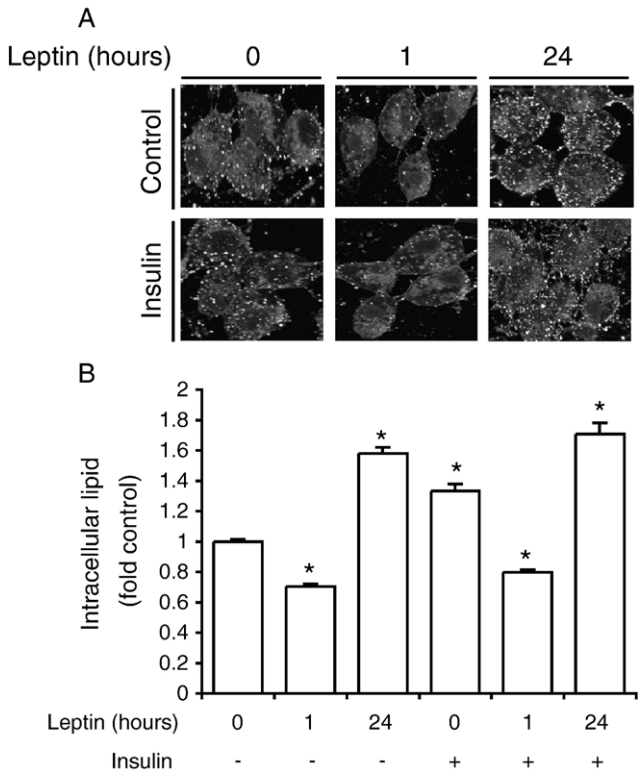

Fig. 6. Regulation of intracellular lipid accumulation. We examined the effect of pretreating HL-1 cells with leptin (60 nmol/L) for 1 or 24 hours in the absence or presence of insulin (100 nmol/L, 3 hours) on intracellular lipid accumulation. Shown in A are representative images showing lipid staining, and the quantitative analysis of these data shown in B represent mean ± SEM of 4 experiments (with total analysis of >50 cells from several distinct fields of view). *$P < .05$, significant with respect to control.

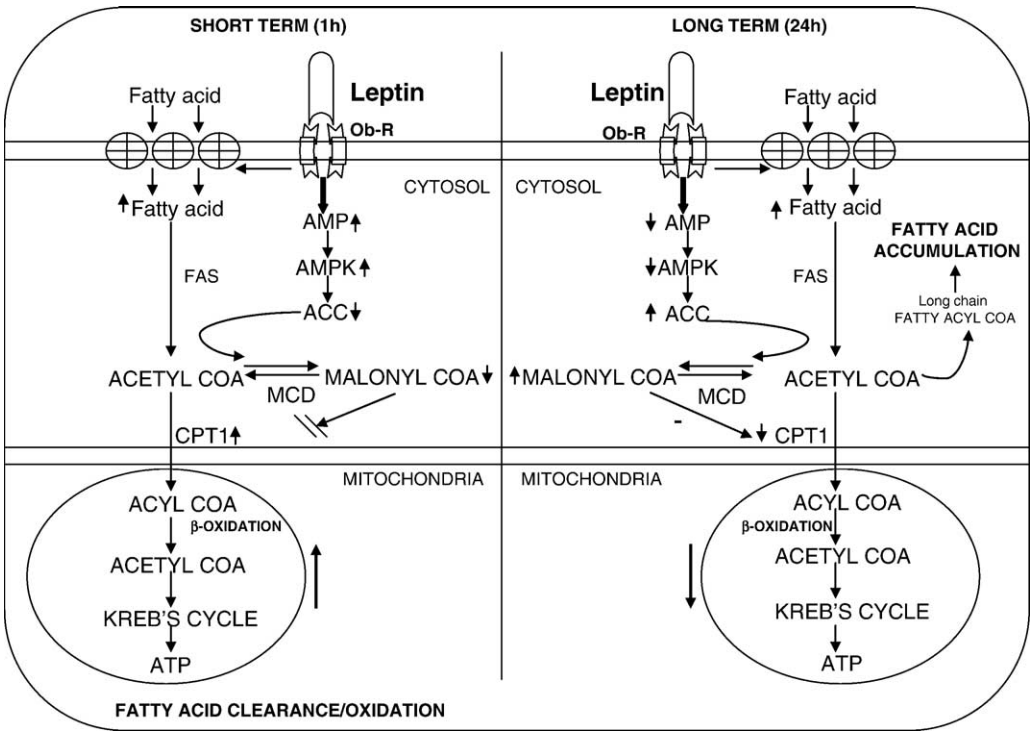

Fig. 7. Schematic representation of the effects of leptin on fatty acid uptake and metabolism in HL-1 cardiomyocytes. Short term (1 hour) treatment increased both fatty acid uptake and oxidation, the latter via increasing AMPK and ACC phosphorylation, which decreases malonyl-coA levels, relieving inhibition of CPT-1 and allowing enhanced oxidation levels. After 24-hour treatment we found increased uptake, but depressed oxidation of fatty acids leading to intracellular lipid accumulation. MCD indicates malonyl-coA decarboxylase, FAS, fatty acyl-CoA synthase; Ob-R, leptin receptor.

metabolic changes associated with heart failure, and the most appropriate treatment, is dependent upon the widespread pathogenesis and various stages of HF that occur. Because long-chain fatty acids are the major energy substrate for cardiomyocytes and imbalance in their uptake and oxidation may elicit lipotoxic effects, it is critical to learn how their metabolism is regulated by hormones whose level changes in obese individuals.

Although leptin is able to directly act on peripheral tissues to regulate energy metabolism [30], very little is known about its ability to modulate cardiac metabolism. Here we focused on examining the effects of leptin on both glucose and fatty acid uptake and metabolism in cardiomyocytes over long and short exposure times. We conducted this study in the HL-1 murine cardiac myocyte cell line [31]. Unlike primary cultures, these cells can be passaged indefinitely in culture and they also remain in a mitotic state typical of immature cardiac myocytes while retaining a mature cardiac myocyte phenotype [31]. Cardiac characteristics of HL-1 cells include an ultrastructure similar to primary cultures of cardiac myocytes, cytoplasmic reorganization, and myofibrillogenesis similar to that observed in mitotic cardiomyocytes in the developing heart, presence of highly ordered myofibrils, the ability to undergo spontaneous contraction, expression of cardiac-specific genes, and the presence of several cardiac-specific voltage-dependent characteristics [31]. Furthermore, transplantation studies suggest HL-1 cells can act as a functional equivalent of endogenous adult cardiomyocytes [32]. Hence, these cells represent an excellent model to elucidate the effects of leptin on cardiomyocyte metabolism.

Here we have established that both short- and long-term exposure of HL-1 cardiomyocytes to leptin increased long-chain fatty acid (LCFA) uptake. This is in keeping with the fact mentioned above that obese and diabetic individuals, who tend to be hyperleptinemic, exhibit increased rates of LCFA utilization. Indeed, alterations in the kinetics of oleate uptake have been observed previously in cardiomyocytes from obese models, with obese or diabetic Zucker rats having an increased V_{max} and K_m when compared with control Wistar rats and an increased V_{max} when compared with lean Zucker rats [33]. Many studies have demonstrated that changes in CD36 expression or localization play a major role in determining the extent of fatty acid uptake in myocytes [34,35]. Stimuli such as insulin and contraction induce the translocation of FAT/CD36 to the cell surface and increase fatty acid uptake [36]. In this study we demonstrate that leptin increased CD36 translocation as well as increased protein content of CD36 and FATP-1 in HL-1 cardiomyocytes. Increased FATP-1 or CD36 expression was also observed in *ob/ob* mice or obese Zucker rats compared with lean littermates [37,38], and it was suggested that this led to intracellular lipid accumulation in these animals, an observation also made in our study.

Intracellular lipid accumulation has widespread cellular consequences, including causing insulin resistance, apopto-

sis, and fibrosis [37,39]. Thus, proper regulation of fatty acid uptake and metabolism is critical for optimal cardiac function. The AMPK/ACC axis is commonly viewed as the major determinant of fatty acid metabolism [40] and, accordingly, was investigated here. It is also interesting to note that one study suggested leptin can alter metabolism in the heart via mechanisms independent of AMPK/ACC [12]. Transport of fatty acids into mitochondria, via carnitine palmitoyl transferase 1 (CPT-1), is the rate-limiting step in fatty acid oxidation. Carnitine palmitoyl transferase 1 is under inhibitory control by malonyl-CoA, which in turn is regulated by ACC and malonyl-CoA decarboxylase. Activation of AMPK results in phosphorylation and inactivation of ACC, and it is known that cardiomyocytes express the 280-kd isoform of ACC [41], an observation confirmed by our results here in HL-1 cells. We investigated changes in ACC phosphorylation in response to leptin treatment by Western blotting using a phospho-specific antibody and found increased AMPK and ACC phosphorylation after short-term exposure to leptin. The increase in ACC phosphorylation would be expected to inhibit this enzyme, decrease malonyl-CoA levels, and thus relieve inhibition on CPT-1. Indeed, we found that LCFA oxidation was increased after 1 hour of leptin treatment (Fig. 7).

It has been previously proposed that leptin functions in vivo as an antilipotoxic hormone, protecting peripheral tissues, including cardiomyocytes, from excessive lipid accumulation [42]. For example, Unger's group has demonstrated that adenoviral-driven expression of leptin prevents various pathologic abnormalities, including increased triglyceride content, observed in the heart of mice overexpressing acyl-CoA synthase specifically in cardiomyocytes [42]. In this study by Lee et al [42], hyperleptinemia was generated by leptin administration for 8 weeks, whereas in our in vitro study we have used a maximum of 24-hour exposure to leptin. Furthermore, in an obese individual who is continuously exposed to a high plasma leptin concentration, fatty acid oxidation is known to be elevated. In short-term (1 hour) experiments, we observed that despite also increasing LCFA uptake, leptin activated fatty acid oxidation and inhibited intracellular lipid accumulation. After longer exposure of 24 hours, phosphorylation of AMPK and ACC was similar to control, and increased lipid accumulation was observed in our study (Fig. 7). It is pertinent to consider the question of whether some differences in the effects of leptin in vitro and in obese humans and rodent models come from the development of leptin resistance in the heart of an obese individual, as has been proposed [43,44]. Alternatively, as alluded to by Lee et al in their study, the lower myocardial lipid level they observed may be a result of leptin's ability to act on other tissues to lower plasma triglyceride and free fatty acid levels.

Glucose metabolism is often impaired in obese individuals due to their inability to respond to changes in the plasma insulin concentration because of insulin resistance [30]. Here, we investigated the effect of leptin on cardiomyocyte insulin sensitivity and observed no effect on regulation of glucose uptake by insulin upon either short- or long-term leptin treatment. Although often considered to play only a minor role in myocardial energy substrate metabolism, glycogen metabolism has been shown to contribute significantly to overall myocardial ATP production [45]. In adequately perfused cardiomyocytes, exogenous glucose is transported into the myocyte and primarily enters the glycolytic pathway or is stored as glycogen. However, under hypoxic conditions when fatty acid and glucose oxidation are decreased, ATP generation from anaerobic glycolysis increases, a phenomenon shown to have a major impact on postischemic recovery [46]. In our study we found that leptin did not alter regulation of glycogen synthesis or glucose oxidation in response to insulin. As mentioned above, it has been documented that cardiomyocyte glucose transport, glycolysis, and glucose oxidation decrease, whereas fatty acid uptake and oxidation increase in obesity and diabetes [14,25]. Again, in vitro and in vivo observations may differ for a variety of reasons including contribution from other hormones in the in vivo milieu, contribution from central nervous system innervation, and the potential for selective leptin resistance as discussed above.

In summary, we demonstrate that leptin immediately increased fatty acid uptake and oxidation in murine cardiomyocytes, yet oxidation decreased over time leading to intracellular lipid accumulation, which may lead to lipotoxic consequences such as apoptosis in heart failure. Leptin did not alter basal or insulin-stimulated glucose uptake or metabolism. These studies that establish the direct effects of leptin on energy metabolism in cardiomyocytes provide insight to the potential role of this hormone in obesity-related heart failure.

Acknowledgment

This work was supported by an operating grant to GS from the Canadian Institutes of Health Research, Institute of Nutrition, Metabolism and Diabetes. GS holds a Canadian Diabetes Association Scholarship in honor of the late Mary A. Bodington. IC would also like to thank the Canadian Institutes of Health Research for support.

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