

Sensitivity of Cardiac Carnitine Palmitoyltransferase to Malonyl-CoA Is Regulated by Leptin: Similarities with a Model of Endogenous Hyperleptinemia

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Acute leptin increase as well as endogenous hyperleptinemia evoked by high-fat diets (HF) activate fatty acid metabolism in nonadipose tissues. This supports the notion that hyperleptinemia is pivotal to prevent/delay steatosis during periods of positive energy balance. We have previously shown that long-term HF spares ectopic accumulation of lipids specifically in the myocardium. Because carnitine palmitoyltransferase I (CPT-I) allows mitochondrial uptake/oxidation of fatty acids, we have hypothesized that leptin drives cardiac CPT-I activity. In the current study, hyperleptinemia was induced in C57BL/6J mice either by exogenous leptin administration or by means of HF, and the ability of malonyl-coenzyme A (malonyl-CoA) (the main endogenous inhibitor of CPT-I) to inhibit cardiac CPT was analyzed. IC_{50} values of malonyl-CoA were $8.1 \pm 1.5 \mu\text{mol/liter}$ in controls vs. $69.3 \pm 5.2 \mu\text{mol/liter}$ ($P < 0.01$) in leptin-treated mice. This effect was also observed in cardiac explants incubated with leptin and was blocked by triciribine, a compound shown to inhibit protein kinase B (Akt) phosphorylation (pAkt). In accordance, acute leptin evoked an increase of cardiac pAkt levels, which correlated with CPT sensitivity to malonyl-CoA. Otherwise, the inhibitory effect of malonyl-CoA was hindered in HF hyperleptinemic mice, and in this case, pAkt levels also correlated with CPT sensitivity to malonyl-CoA. Our data show that leptin reduces the sensitivity of cardiac CPT-I to malonyl-CoA and suggest the involvement of an Akt-related signaling pathway in this effect. This mechanism appears to be sensitive to both acute and chronic hyperleptinemia. We conclude that this action of leptin is pivotal to drive cardiac metabolism under situations associated to hyperleptinemia. (*Endocrinology* 151: 1010–1018, 2010)

Cardiac energetic needs are met by mitochondrial oxidation of fatty acids (FA) and, in minor extent, of pyruvate. The relative contribution of each fuel is driven by FA availability and is linked to the type of diet, physical activity, or pathophysiological conditions such as diabetes, hypertrophy, or chronic ischemia. Control of FA oxidation largely depends on mitochondrial uptake of FA by the carnitine palmitoyltransferase (CPT) system, which

includes the sequential action of CPT-I and CPT-II. The transfer of FA moiety from acyl-coenzyme A (acyl-CoA) to carnitine is carried out by CPT-I. CPT-II accounts for the transport of acylcarnitine derivatives into the mitochondria and subsequent transference of acyl residues to CoA, yielding intramitochondrial acyl-CoA (1–4).

CPT-I activity is competitively inhibited by malonyl-CoA and is affected by alterations of the nutritional status,

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Abbreviations: ACC, Acetyl-CoA carboxylase; acyl-CoA, acyl-coenzyme A; Akt, protein kinase B; AMPK, AMP-activated protein kinase; CPT, carnitine palmitoyltransferase; DIO, diet-induced obesity; FA, fatty acids; HF, high fat; LF, low fat; pAkt, phosphorylated Akt; pAMPK, phosphorylated AMPK; pSTAT3, phosphorylated signal transducer and activator of transcription 3; TCB, triciribine.

such as starvation or changes of diet composition (5–7). Synthesis of malonyl-CoA is regulated by the AMP-activated protein kinase (AMPK)/acetyl-CoA carboxylase (ACC) pathway, depending on energetic needs (8). Regulation of cardiac FA oxidation by AMPK occurs through phosphorylation and inhibition of ACC. Thus, when ATP supply decreases, AMPK is phosphorylated, ACC is inhibited, malonyl-CoA levels fall, and β -oxidation is activated. Under these conditions, pyruvate oxidation is inhibited (9) and FA become almost the unique source of oxidative ATP production (10). In contrast, when energetic needs are fulfilled, dephosphorylation of AMPK leads to the activation of ACC, which stimulates malonyl-CoA synthesis and drives long chain acyl-CoA toward complex lipid synthesis. If this situation lasts for a long period, ectopic deposition of lipids and cardiac lipotoxicity can occur (11–13). Cardiac AMPK is also negatively regulated by protein kinase B (Akt) (14).

The influence of diet composition on cardiac metabolism depends on the partial contribution of fat/carbohydrates to the daily caloric intake. In the heart, short-term treatment with high-fat (HF) diets (8 wk) has been shown to evoke the adaptation of energetic metabolism, characterized by increased phosphorylation of AMPK and mitochondrial uncoupling. This adaptation aims at preventing ectopic lipid deposition, and it has been proposed that leptin would play a pivotal role in this cardiac metabolic remodelling (15). We have also reported that long-term treatment (32 wk) with a HF diet, which leads to severe obesity and marked hyperinsulinemia/hyperleptinemia, spares triglyceride accumulation in the heart. Although under these conditions leptin resistance affects most of tissues/organs, cardiac leptin receptors keep full responsiveness, suggesting that leptin plays a role in preventing lipid accumulation (16). Our hypothesis is that leptin might drive FA metabolism in cardiac tissue by modulating CPT-I activity. Therefore, the current study has been designed to characterize the effect of hyperleptinemia on CPT activity. With this purpose, C57BL/6J were made hyperleptinemic either by administration of exogenous leptin or by a dietary treatment with HF chow, and we analyzed the ability of malonyl-CoA to inhibit CPT under these conditions. Our goal was to provide insights on mechanisms involved in preventing/delaying cardiac steatosis under pathophysiological conditions triggered by long-term overload with dietary fat.

Materials and Methods

Experimental design

In this study, we have characterized the effect of 1) diet-induced obesity (DIO)/hyperleptinemia, and 2) leptin on the sen-

sitivity of cardiac CPT to malonyl-CoA. Four-week-old male C57BL/6J mice (Harlan, Barcelona, Spain) were housed (five per cage) under a 12-h light, 12-h dark cycle in a temperature-controlled room (22 C) with food and water *ad libitum*. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996). Animals were divided into two groups with similar average body weight, housed five per cage, and assigned either to a low-fat (LF) or to a HF diet. LF (D12450B, 10 kcal % fat, 70 kcal % carbohydrates and 20 kcal % protein; 3.85 kcal/g) or HF (D12451, 45 kcal % fat, 35 kcal % carbohydrates and 20 kcal % protein; 4.73 kcal/g) diets were supplied by Research Diets, Inc. (New Brunswick, NJ) and will be referred to as LF and HF, respectively. After 32-wk dietary treatment, groups of animals were treated (ip) either with saline or 1 mg/kg mouse leptin (Sigma, St. Louis, MO) at 0900 h. Ninety minutes later, animals were decapitated, blood collected in chilled EDTA-coated polypropylene tubes, and left ventricle dissected. Animals had always free access to food and tap water. Plasma samples were stored at -80°C until assay, and tissues were prepared for CPT activity quantification. Some hearts from LF mice were used to measure CPT activity in left ventricle explants. Dosage of leptin was chosen in basis to previous studies of our group (15).

Plasma measurements

Plasma leptin concentration was analyzed by using a specific RIA kit for murine leptin (Linco Research, Inc., St. Charles, MO) (4.9% intraassay variation, 3.3% interassay variation). Insulin was determined by means of a specific enzyme immunoassay kit for mouse insulin (Mercodia, Denmark) (2.2% intraassay variation, 4.9% interassay variation).

Measurement of cardiac triglycerides

Triglyceride content in heart was determined as previously described (15). Briefly, 20 mg of wet tissue were homogenized in a solvent mixture containing 40 μl of 2 mM NaCl/20 mM EDTA/50 mM sodium phosphate buffer (pH 7.4), 40 μl of tert-butanol, and 20 μl of Triton X-100/methanol mixture (1/1). Triglycerides were measured with a Sigma diagnostic kit.

Preparation of left ventricle explants

Left ventricles were sliced (250 μm thick) with a tissue chopper. Each slice was incubated in 1 ml oxygenated (95% O_2 -5% CO_2) Krebs-Henseleit solution containing 10 $\mu\text{g}/\text{ml}$ leptin. After 1-h incubation at 37 C, slices were processed for CPT assay, as described below.

CPT assay

Total CPT activity was measured in isolated mitochondria by means of a colorimetric method (17, 18). Briefly, tissues (10% wt/vol) were homogenized, at 4 C, in 20 mM HEPES (pH 7.4) containing 140 mM KCl, 10 mM EDTA, and 5 mM MgCl_2 . The homogenate was centrifuged at $500 \times g$ (10 min, 4 C) and the supernatant recentrifuged at $9000 \times g$ (35 min, 4 C). The pellet was collected and resuspended in 150 μl buffer. Isolated mitochondria (0.1 mg protein/ml) were assayed in 100 μl assay buffer containing 20 mM HEPES (pH 7.4), 1 mM EGTA, 220 mM sucrose, 40 mM KCl, 0.1 mM 5,5'-dithio-bis(2-nitrobenzoic) acid (Sigma), 1.3 mg/ml BSA, and 40 μM palmitoyl-CoA (Sigma) at 25 C. The reaction was started by adding 1 mM carnitine and mon-

itored at 412 nm (VERSAmix Microplate Reader; Molecular Devices, Sunnyvale, CA) after 10-min incubation at 25 C. Inhibition assays with malonyl-CoA (0–100 μ mol/liter) were performed under identical experimental conditions but malonyl-CoA was added just before carnitine. CPT activity in absence/presence of malonyl-CoA will be further referred as total CPT and malonyl-CoA-insensitive CPT activities, respectively. Results are expressed as Δ Abs/min/mg protein.

Western blot analysis for the phosphorylated forms of AMPK (pAMPK), signal transducer and activator of transcription 3 (pSTAT3), and Akt (pAkt)

pAMPK, pSTAT3, and pAkt were measured in whole cardiac left ventricle. Briefly, tissues were homogenized in ice-cold buffer containing 0.42 M NaCl, 20 mM HEPES (pH 7.9), 1 mM $\text{Na}_4\text{P}_2\text{O}_7$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 20% glycerol, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 20 mM sodium fluoride, 1 mM trisodium orthovanadate, and 2 mM phenylmethylsulfonyl fluoride. Tubes containing homogenates were frozen at -80°C and thawed at 37°C three consecutive times, then centrifuged for 10 min at 4°C . Equivalent amounts of proteins (50 μ g) present in the supernatant were loaded in Laemli buffer [50 mM Tris (pH 6.8), 10% sodium dodecyl sulfate, 10% glycerol, 5% mercaptoethanol, and 2 mg/ml blue bromophenol] and size-separated in 15% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes (Amersham Pharmacia, Barcelona, Spain) using a transblot apparatus (Bio-Rad, Madrid, Spain). For immunoblotting, membranes were blocked with 5% nonfat dried milk in Tween-PBS for 1 h. Primary antibodies against pAMPK- α (Thr¹⁷²) (1:1000 final dilution; Cell Signaling Technology, Beverly, MA), AMPK- α (1:1000 final dilution; Cell Signaling Technology), pSTAT3 (Tyr⁷⁰⁵) (1:1000 final dilution; Cell Signaling Technology), STAT3 (1:1000 final dilution; Santa Cruz Biotechnology, Santa Cruz, CA), pAkt (Ser⁴⁷³) (1:100 final dilution; Cell Signaling Technology), and AKT (1:100 final dilution; Cell Signaling Technology) were applied at the convenient dilution overnight at 4°C . After washing, appropriate secondary antibodies (antirabbit IgG-peroxidase conjugated) were applied for 1 h at a dilution of 1:5000. Blots were washed, incubated in commercial enhanced chemiluminescence reagents (Amersham Pharmacia) and exposed to autoradiographic film. Films were scanned by using a GS-800 Calibrated Densitometer (Bio-Rad), and blots were quantified using Quantity One software (Bio-Rad). Values for pAMPK, pSTAT3, and pAkt were normalized with AMPK, STAT3, and Akt, respectively.

Triciribine (TCB) treatment protocol

TCB (BioMol, Plymouth Meeting, PA) was administered by i.p. as described by Shein *et al.* (19). Briefly, TCB was dissolved in 2% dimethylsulfoxide/saline at a final concentration of 0.1 mg/ml. TCB or vehicle (2% dimethylsulfoxide in saline) administration was conducted at 0900 h at 1 mg/kg. Thirty minutes later, leptin (1 mg/kg) or saline were administered by i.p., animals were killed at 1100 h, and cardiac mitochondria immediately prepared for CPT activity assay. Malonyl-CoA was tested at 50 μ mol/liter.

Statistics

Variations of CPT activity in response to leptin and malonyl-CoA were analyzed by a two-way ANOVA. Other parameters

TABLE 1. Effect of HF diet on body weight, heart weight, cardiac triglycerides (TG), and plasma parameters

	LF diet	HF diet
Body weight (g)	34.5 \pm 0.5	48.8 \pm 1.1 ^a
Heart weight (mg)	162.5 \pm 2.8	171.3 \pm 2.4 ^b
Cardiac TG (mg/g tissue)	39.4 \pm 5.1	27.9 \pm 2.7
Plasma leptin (ng/ml)	13.6 \pm 1.7	28.4 \pm 5.9 ^b
Plasma insulin (μ g/liter)	2.10 \pm 0.31	5.81 \pm 0.59 ^a

^a $P < 0.001$; ^b $P < 0.05$ compared vs. LF group (Newman Keuls' test).

were analyzed by a one-way ANOVA, followed by Newman-Keuls' *post hoc* test. Statistical significance was set at $P < 0.05$.

Results

Effect of dietary treatment on cardiac triglycerides and plasma parameters

Table 1 summarizes the effect of dietary treatment on body and heart weight, cardiac triglyceride content, and biochemical plasma parameters. Animals exposed to HF diet exhibited significant overweight ($F_{(1,32)} = 94.398$; $P < 0.001$), as well as elevated leptin ($F_{(1,15)} = 6.319$; $P < 0.05$) and insulin plasma concentration ($F_{(1,16)} = 30.368$; $P < 0.001$). We also detected a slight increase of heart weight ($F_{(1,30)} = 5.24$; $P < 0.05$), although cardiac triglyceride content was not affected by the type of diet.

Leptin reduces the inhibitory effect of malonyl-CoA on cardiac CPT activity both *in vivo* and *in vitro*

We first determined the ability of malonyl-CoA to inhibit cardiac CPT in isolated mitochondria of mice treated with exogenous leptin. Figure 1A illustrates the effect of leptin administration (1 mg/kg) on cardiac CPT inhibition by malonyl-CoA (10–100 μ mol/liter). Two-way ANOVA revealed that the effect of malonyl-CoA ($F_{(1,44)} = 18,420$; $P < 0.001$) was dependent on leptin treatment ($F_{(1,44)} = 2,712$; $P < 0.05$, for the interaction between malonyl-CoA and leptin). IC_{50} values of malonyl-CoA were 8.1 ± 1.5 μ mol/liter in control animals *vs.* 69.3 ± 5.2 100 μ mol/liter ($P < 0.01$) in leptin-treated mice. Plasma leptin concentrations were 13.6 ± 1.7 ng/ml in control animals *vs.* 81.1 ± 12.8 ng/ml ($P < 0.01$) in animals treated with exogenous leptin.

The effect of leptin on CPT activity was also tested in left ventricle explants incubated with murine leptin (Fig. 1B). We observed that 50 μ mol/liter malonyl-CoA inhibited CPT activity (2-way ANOVA, $F_{(1,22)} = 25,658$; $P < 0.001$), and this effect was abolished when explants were previously incubated with 10 μ g/ml leptin ($F_{(1,22)} = 4,290$; $P < 0.05$ for the interaction between malonyl-CoA and

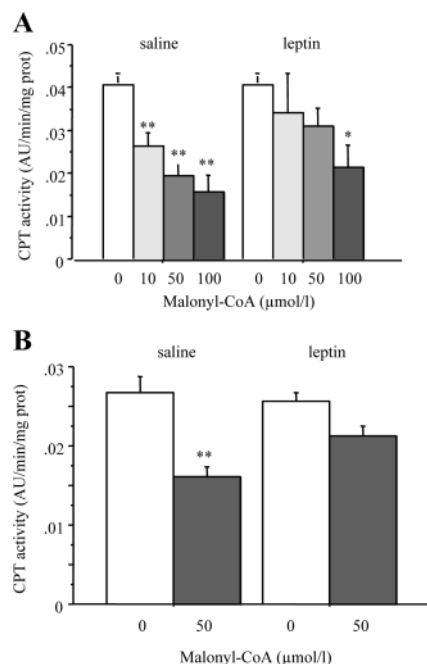


FIG. 1. Effect of leptin on CPT inhibition by malonyl-CoA. A, Effect of 1 mg/kg leptin on cardiac CPT inhibition by malonyl-CoA (0–100 μmol/liter). Left ventricle mitochondria (0.1 mg protein/ml) were incubated for 10 min with malonyl-CoA and CPT activity monitored spectrophotometrically at 412 nm. In the control group, significant inhibition of CPT was detected from 10 μmol/liter malonyl-CoA. In leptin-treated mice, inhibition was only detected at 100 μmol/liter malonyl-CoA. Values are means ± SEM of six to eight animals. *, $P < 0.05$; **, $P < 0.01$ compared with their respective controls (white columns) (Newman-Keuls' test). B, Effect of 50 μmol/liter malonyl-CoA on CPT activity in heart explants incubated with 10 μg/ml leptin. As illustrated in this figure, malonyl-CoA only reduced CPT activity in controls but not in heart explants incubated with leptin. Values are means ± SEM of six to eight mice. **, $P < 0.01$ compared with the control group (white column) (Newman-Keuls' test).

treatment with leptin). Further statistical analysis indicated that malonyl-CoA only inhibited CPT activity in controls (one-way ANOVA, $F_{(1,11)} = 18,984$; $P < 0.01$), but not in samples incubated with leptin.

TCB, an inhibitor of Akt phosphorylation, antagonizes the effect of leptin in reducing CPT sensitivity to malonyl-CoA

Activity of cardiac CPT was determined in mice receiving 1 mg/kg TCB previous to leptin administration. As illustrated in Fig. 2, the ability of 50 μmol/liter malonyl-CoA to inhibit CPT activity was reduced by 65% in leptin-treated mice (1 mg/kg) and the effect of leptin was fully blocked by TCB ($P < 0.05$).

Leptin enhances Akt and STAT3 phosphorylation in the heart

Because phosphorylation of STAT3 and/or Akt is indicative of activation of signaling pathways coupled to leptin receptors, we measured the effect of 1 mg/kg leptin on both pSTAT3 and pAkt concentration in cardiac tissue.

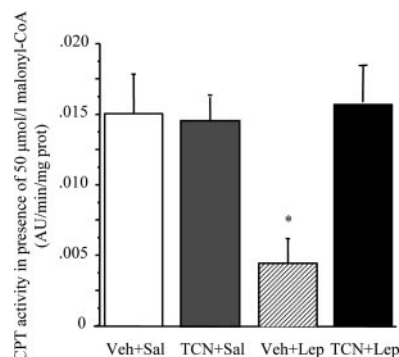


FIG. 2. TCB antagonizes the effect of leptin in reducing the sensitivity of CPT to malonyl-CoA. Mice were treated either with vehicle+saline (white bars), TCB+saline (gray bars), vehicle+leptin (hatched bars), or TCB+leptin (black bars). TCB did not modify the effect of malonyl-CoA on CPT activity (gray bars), but leptin significantly reduced the ability of malonyl-CoA to inhibit CPT (hatched bars). When TCB was administered previously to leptin, the hormone failed to impair CPT inhibition by malonyl-CoA. *, $P < 0.05$ (Newman-Keuls' test).

As illustrated in Fig. 3, pSTAT3 ($F_{(1,17)} = 6,681$; $P < 0.05$) as well as pAkt ($F_{(1,15)} = 5,297$; $P < 0.05$) appeared to be increased in leptin-treated mice.

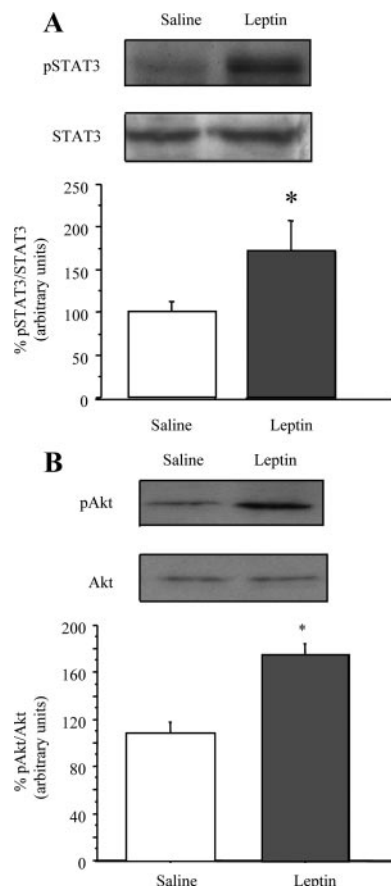


FIG. 3. Effect of 1 mg/kg leptin on STAT3 (A) and Akt phosphorylation in cardiac tissue (B). Values are means ± SEM of 8–10 mice. *, $P < 0.05$ compared with the control group (Newman-Keuls' test).

Plasma leptin concentration positively correlates with pAkt as well as with malonyl-CoA-insensitive CPT activity

Although it is widely assumed that elevated concentrations of malonyl-CoA inhibit CPT-I and preserve CPT-II activity, sensitivity of CPT-I to malonyl-CoA is modulated by several factors. We have reconsidered the idea that CPT activity in presence of malonyl-CoA reflects only CPT-II activity, as previously suggested by Kim *et al.* (5). In this paper, we refer to total CPT activity (measured in absence of malonyl-CoA) and malonyl-CoA insensitive CPT activity (determined in presence of malonyl-CoA).

Figure 4 shows the correlations between plasma leptin concentration and pAkt (Fig. 4A) or malonyl-CoA insen-

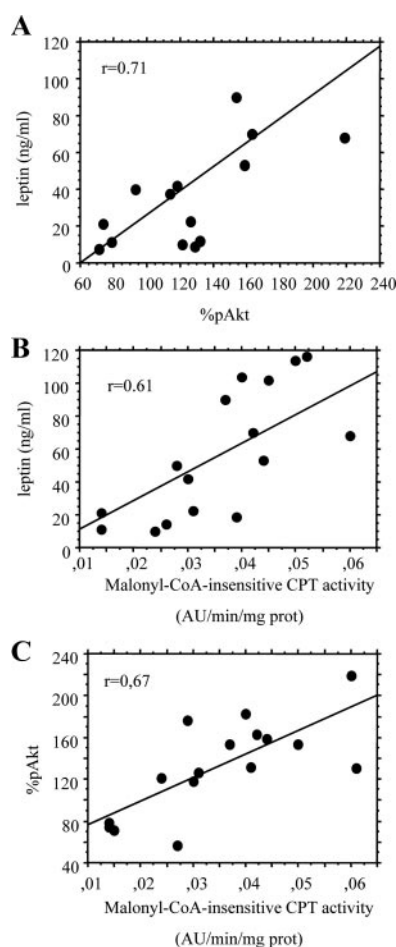


FIG. 4. A, Correlation between plasma leptin concentration and pAkt in hearts from normal mice receiving 1 mg/kg leptin (ip) or vehicle. Simple regression analysis evidences a positive correlation between leptin and pAkt, which suggest that leptin activates signaling pathways in the heart leading to an increase of pAkt. B, Correlation between plasma leptin and CPT activity in presence of 50 $\mu\text{mol/liter}$ malonyl-CoA (malonyl-CoA-insensitive CPT activity) determined in cardiac mitochondria from mice receiving 1 mg/kg leptin (ip) or vehicle. This correlation reveals that the effect of malonyl-CoA appears to be hindered when plasma leptin levels are elevated. C, Correlation between pAkt and CPT activity in presence of malonyl-CoA. This correlation suggests that Akt phosphorylation is coincident with a decreased ability of malonyl-CoA to inhibit CPT.

sitive CPT activity (Fig. 4B). First, we observed that Akt phosphorylation increased proportionally to plasma leptin concentration ($F_{(1,16)} = 16,022$; $P < 0.01$; Fig. 4A). Because leptin reduces the ability of malonyl-CoA to inhibit CPT, we also analyzed, by simple regression analysis, the correlation between plasma leptin concentration and malonyl-CoA insensitive CPT activity. As illustrated in Fig 4B, plasma leptin concentration positively correlated with CPT activity ($F_{(1,16)} = 7,387$; $P < 0.05$) in samples exposed to 50 $\mu\text{mol/liter}$ malonyl-CoA. Simple regression analysis revealed that pAkt was also proportional to malonyl-CoA-insensitive CPT activity ($F_{(1,15)} = 16,348$; $P < 0.01$; Fig. 4C). All correlations correspond to individuals receiving either saline or 1 mg/kg leptin (ip).

Otherwise, plasma leptin concentration also correlated positively with pSTAT3 levels. Nevertheless, no correlation was detected between pSTAT3 and (data not shown).

Inhibition of cardiac CPT by malonyl-CoA is attenuated in mice receiving a HF diet

We tested the ability of 50 $\mu\text{mol/liter}$ malonyl-CoA to inhibit CPT activity in isolated mitochondria of both in normal and HF mice. As illustrated in Fig. 5A, two-way ANOVA revealed a significant effect of both dietary treat-

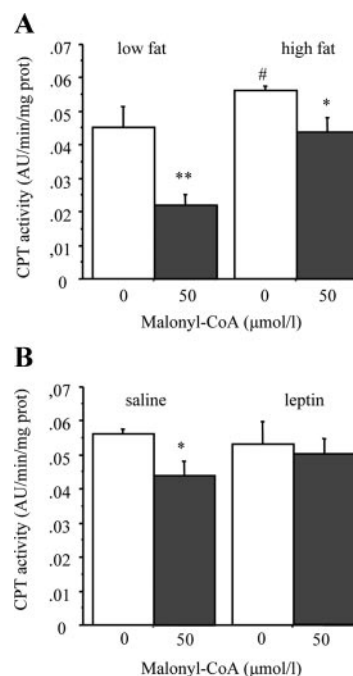


FIG. 5. A, Effect of malonyl-CoA on cardiac CPT activity in mice fed a HF diet during 32 wk. The 50 $\mu\text{mol/liter}$ malonyl-CoA decreased 55% total CPT activity in normal mice but only 21% in HF mice. Values are means \pm SEM of five to seven samples. *, $P < 0.05$; **, $P < 0.01$ compared with their respective control groups; #, $P < 0.05$ compared with the LF group in absence of 50 $\mu\text{mol/liter}$ malonyl-CoA (Newman-Keuls' test). B, Treatment with leptin fully prevents the inhibitory effect of malonyl-CoA on CPT activity in HF mice. Values are means \pm SEM of five to seven samples. *, $P < 0.05$ compared with their respective control group (Newman-Keuls' test).

ment ($F_{(1,18)} = 15,417$; $P < 0.001$) and malonyl-CoA ($F_{(1,18)} = 18,448$; $P < 0.001$). Malonyl-CoA inhibited CPT activity both in control (one-way ANOVA, $F_{(1,8)} = 10,699$; $P < 0.01$) and in HF mice (one-way ANOVA, $F_{(1,10)} = 6,757$; $P < 0.05$). Nevertheless, the inhibition detected in normal animals was significantly more pronounced than that detected in HF mice (one-way ANOVA, $F_{(1,9)} = 14,104$; $P < 0.01$). In the control group, malonyl-CoA decreased almost 55% of total CPT activity, which theoretically corresponds to the contribution of CPT-I to total CPT activity. In HF mice, inhibition was approximately 21%, which suggests a loss of sensitivity of CPT-I to inhibition by malonyl-CoA. This residual effect of malonyl-CoA was not observed in HF mice receiving 1 mg/kg leptin. In this case, two-way ANOVA revealed an effect of malonyl-CoA ($F_{(1,18)} = 8,543$; $P < 0.01$), which was significant in controls (one-way ANOVA, $F_{(1,10)} = 6,757$; $P < 0.05$) but not in leptin-treated mice. In this case, plasma leptin concentrations were 28.4 ± 5.9 ng/ml in control animals *vs.* 82.5 ± 11.8 ng/ml ($P < 0.01$) in animals treated with exogenous leptin (Fig. 5B).

pAkt is increased by HF diet

Dietary treatment with HF increased cardiac Akt phosphorylation, as appears illustrated in Fig. 6A ($F_{(1,18)} = 12,413$; $P < 0.01$). Levels of pAkt positively correlated with plasma leptin concentration ($F_{(1,9)} = 7,705$; $P < 0.05$; Fig. 6B) and also with malonyl-CoA insensitive CPT activity ($F_{(1,8)} = 8,571$; $P < 0.05$; Fig. 6C). Dietary treatment with HF reduced on basal levels of pAMPK ($F_{(1,17)} = 9,783$; $P < 0.01$), which were determined by Western blot analysis (Fig. 7A). Because phosphorylation of AMPK has been shown to be negatively regulated by pAkt, we also analyzed the correlation between pAkt and pAMPK. As illustrated in Fig. 7B, the concentration of pAkt negatively correlated with pAMPK levels ($F_{(1,18)} = 18,980$; $P < 0.001$).

Discussion

The major finding of the current study refers to the involvement of circulating leptin in regulating the sensitivity of cardiac CPT to its main endogenous inhibitor, malonyl-CoA (Fig. 1A). Moreover, this leptin effect seems to be linked to the activation of Akt, because we have observed that 1) leptin administration increases myocardial content of pAkt (Fig. 3B), and 2) inhibition of Akt by TCB antagonizes the effect of leptin in reducing the sensitivity of CPT to malonyl-CoA. The shift of malonyl-CoA IC_{50} observed in leptin-treated mice can theoretically reflect either a direct or an indirect effect of the hormone. In fact, a central

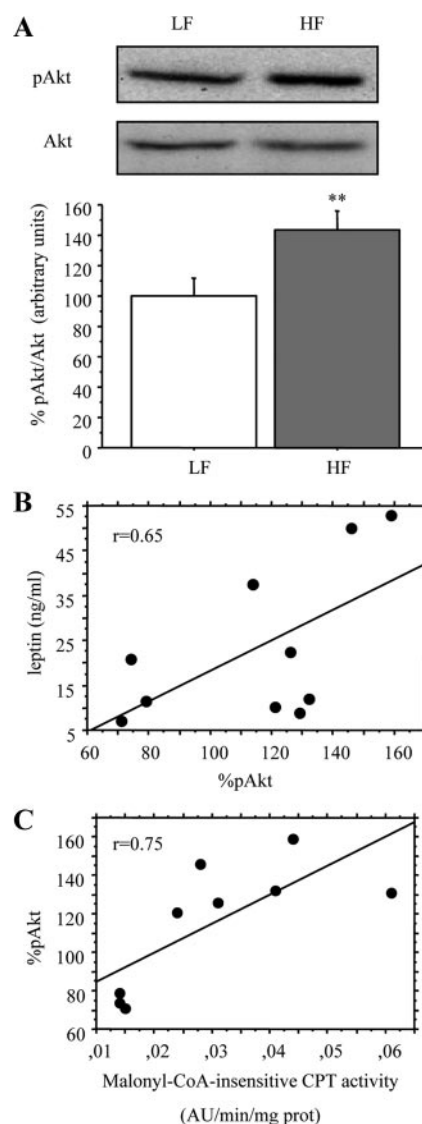


FIG. 6. A, Influence of dietary treatment on cardiac Akt phosphorylation. Both Akt and pAkt were measured by Western blot analysis in left ventricle. Diagram bars show the result of densitometric analysis of Akt immunoblots, expressed as percentage of pAkt in the control group. Values are means \pm SEM of eight to nine samples. **, $P < 0.01$ compared with controls (Newman-Keuls' test). B, Correlation between plasma leptin concentration and pAkt in mice undergoing a dietary treatment with a HF diet during 32 wk. Regression analysis shows a positive correlation between plasma leptin concentration and pAkt, as also observed in normal mice treated with exogenous leptin. C, Correlation between pAkt and CPT activity in presence of malonyl-CoA.

action of leptin might activate autonomous responses (20), leading to the stimulation of cardiac β -adrenergic receptors leading to Akt phosphorylation (21). Nevertheless, because leptin was also effective in heart explants (Fig. 1B), we suggest that, in spite of a hypothetical contribution of central mechanisms, the hormone acts directly on cardiac leptin receptors. Altogether these results point to a pivotal role of leptin in controlling FA catabolism in the heart.

Minokoshi *et al.* (22) have demonstrated that leptin facilitates β -oxidation by stimulating the AMPK/ACC

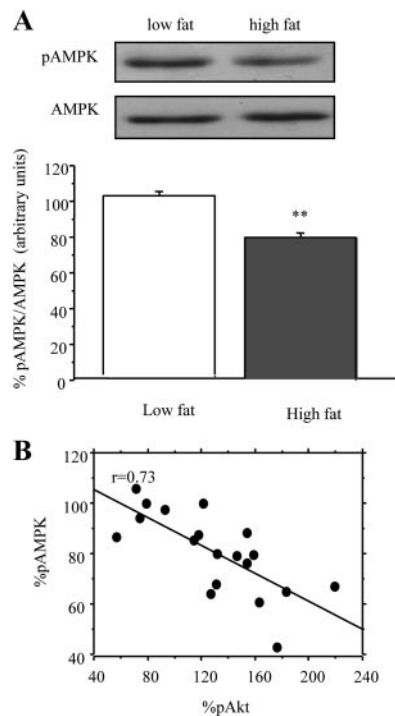


FIG. 7. Influence of dietary treatment on cardiac AMPK phosphorylation. **A**, Cardiac expression of AMPK and pAMPK measured by Western blot analysis. Diagram bars show the result of densitometric analysis of AMPK immunoblots, expressed as percentage of pAMPK in the control group. Values are means \pm SEM of eight to nine samples. **, $P < 0.01$ compared with low-fat group (Newman-Keuls' test). **B**, Negative correlation between pAMPK and pAkt.

pathway in skeletal muscle cells and by reducing malonyl-CoA synthesis. Our current data emphasize the existence of an alternative leptin-mediated pathway which would enhance β -oxidation by decreasing the sensitivity of CPT-I to malonyl-CoA. Concerning the underlying mechanism downstream of leptin receptors, a number of data point to the involvement of Akt. First, although both pAkt and pSTAT3 were increased by leptin in cardiac tissue, malonyl-CoA-insensitive CPT activity only correlated with pAkt levels (Fig. 4C). Second, plasma leptin concentrations in HF hyperleptinemic mice also correlated with pAkt levels which, in turn, were proportional to malonyl-CoA-insensitive CPT activity. Third, the effect of leptin on CPT kinetics was abolished by TCB, an inhibitor of Akt phosphorylation (23) which has been previously shown to inhibit Akt *in vivo* (19). Hence, a sequential activation of Akt and CPT might account for the effect of leptin. Our data strongly suggest that leptin-evoked phosphorylation of Akt is integral to mechanisms regulating β -oxidation and, in accordance with Atkinson *et al.* (24), point to the relevance of leptin in cardiac FA oxidation by an AMPK-independent mechanism. Although a detailed mechanism cannot be drawn from our data, we speculate that pAkt-evoked phosphorylation of CPT-I at Ser/Thr residues might regulate the affinity of malonyl-CoA for CPT-I. In

fact, Akt is a Ser/Thr kinase and CPT-1 has been shown to be a substrate of these enzymes (23–27).

A main question that emerges from our study concerns the physiological relevance of a leptin-dependent regulation of CPT-I. As plasma leptin values after administration of 1 mg/kg leptin largely exceed (~ 7 -fold) physiological concentrations of the hormone, even at the zenith of the circadian rhythm, we analyzed if regulation of CPT kinetics by leptin could be actually relevant under physiopathological situations characterized by hyperleptinemia. In accordance, we demonstrate that the sensitivity of cardiac CPT to malonyl-CoA appears to be impaired in mice made hyperleptinemic by means of a dietary treatment providing 45% cal from lard (Fig. 5A). In this study, it was also shown that the small inhibition of CPT activity elicited by malonyl-CoA in HF mice is sensitive to exogenous leptin (Fig. 5B). This supports the notion that cardiac tissue keeps leptin responsiveness even after long-lasting obesogenic dietary treatments (16) and also suggests that leptin might contribute to delay ectopic lipid deposition by regulating CPT-I even after insulin resistance sets up. In a recent paper, Wright *et al.* (28) have reported that myocardial FA utilization appears to be increased after HF feeding by a mechanism compatible with Akt activation. All this suggests that cross talk between leptin and insulin receptors in the heart might constitute a relevant mechanism to delay cardiac steatosis in obesity. At this point, it has to be noted that aleptinemic, and insulin-resistant, *ob/ob* mice exhibit cardiac steatosis, which is sensitive to leptin therapy (29).

The role of AMPK in regulating cardiac metabolism during health and disease has focused a large body of research (10, 30). In a previous study, we have demonstrated that the increase of AMPK phosphorylation is integral to the short-term remodelling of cardiac metabolism evoked by FA overload (15). Nevertheless, in the current work, we have detected a decrease of AMPK phosphorylation in DIO mice (Fig. 7A), together with an increase of pAkt (Fig. 6A). The negative correlation between pAkt and pAMPK (Fig. 7B) is in accordance with previous works demonstrating that cardiac Akt negatively regulates AMPK phosphorylation (14). This suggests that during long-term exposition to HF, pAkt and not pAMPK might drive FA metabolism.

Finally, the role of leptin in regulating CPT activity can be considered from two different perspectives. During periods of elevated caloric intake, which are initially linked to an increase of adiposity and leptinemia and later to obesity and insulin-resistance, leptin might drive FA oxidation and contribute to impair accumulation of lipids in nonadipose tissues (13) and to manage an eventual ATP deficit in insulin resistant hearts. On the other hand, the

effect of leptin might be detrimental in other situations, such as hypertrophic and/or postischemic heart. Under these conditions, high rates of FA oxidation, which inhibit pyruvate oxidation (31), would favor glycogen-related cardiomyopathy (10, 32).

In summary, this study shows that acute leptin reduces CPT-I sensitivity to malonyl-CoA in normal animals and suggests that hyperleptinemia might account for the impaired sensitivity of CPT-I to malonyl-CoA in obese mice through Akt. Our work provides insights to define cardiac metabolic remodeling during DIO, which are necessary to better understand clinical implications of obesity on cardiac disease (2, 33–37). Moreover, we suggest that leptin antagonists might be useful drugs to modulate cardiac metabolism of FA (38, 39).

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