Biochemical Journal (2017) **474** 149–162
DOI: 10.1042/BCJ20160804

PORTLAND PRESS

Research Article

A new leptin-mediated mechanism for stimulating fatty acid oxidation: a pivotal role for sarcolemmal FAT/CD36

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Leptin stimulates fatty acid oxidation in muscle and heart; but, the mechanism by which these tissues provide additional intracellular fatty acids for their oxidation remains unknown. We examined, in isolated muscle and cardiac myocytes, whether leptin, via AMP-activated protein kinase (AMPK) activation, stimulated fatty acid translocase (FAT/CD36)-mediated fatty acid uptake to enhance fatty acid oxidation. In both mouse skeletal muscle and rat cardiomyocytes, leptin increased fatty acid oxidation, an effect that was blocked when AMPK phosphorylation was inhibited by adenine 9-β-D-arabinofuranoside or Compound C. In wild-type mice, leptin induced the translocation of FAT/CD36 to the plasma membrane and increased fatty acid uptake into giant sarcolemmal vesicles and into cardiomyocytes. In muscles of FAT/CD36-KO mice, and in cardiomyocytes in which cell surface FAT/CD36 action was blocked by sulfo-*N*-succinimidyl oleate, the leptin-stimulated influx of fatty acids was inhibited; concomitantly, the normal leptin-stimulated increase in fatty acid oxidation was also prevented, despite the normal leptin-induced increase in AMPK phosphorylation. Conversely, in muscle of AMPK kinase-dead mice, leptin failed to induce the translocation of FAT/CD36, along with a failure to stimulate fatty acid uptake and oxidation. Similarly, when siRNA was used to reduce AMPK in HL-1 cardiomyocytes, leptin failed to induce the translocation of FAT/CD36. Our studies have revealed a novel mechanism of leptin-induced fatty acid oxidation in muscle tissue; namely, this process is dependent on the activation of AMPK to induce the translocation of FAT/CD36 to the plasma membrane, thereby stimulating fatty acid uptake. Without increasing this leptin-stimulated, FAT/CD36-dependent fatty acid uptake process, leptin-stimulated AMPK phosphorylation does not enhance fatty acid oxidation.

Introduction

Leptin, an adipokine released from adipose tissue, is a key regulator of fatty acid metabolism in muscle tissues, since this hormone is known to increase the rate of fatty acid oxidation in skeletal muscle and the heart [1–6]. Although leptin activates many signal transduction pathways (cf. [7]), leptin has been thought to stimulate fatty acid oxidation in muscle tissue primarily via the AMP-activated protein kinase (AMPK)/acetyl-CoA carboxylase 2 (ACC2)/carnitinepalmitoyltransferase 1 (CPTI) axis [3,8,9], while not necessarily excluding the involvement of other signalling pathways [4,5,10].

Despite the considerable evidence that leptin stimulates fatty acid oxidation in muscle tissues, it is not known how the additional fatty acids that are oxidized are delivered into these peripheral tissues. Increased fatty acid provision from adipose tissue may not be required, since, in isolated muscle

Received: 28 August 2016
Revised: 3 October 2016
Accepted: 8 November 2016

Accepted Manuscript online: 8 November 2016
Version of Record published: 22 December 2016

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Biochemical Journal (2017) 474 149–162
DOI: 10.1042/BCJ20160804

[1,2,11] or perfused hearts [4,5], leptin induced an increase in fatty acid oxidation despite the fact that the external fatty acid supply was clamped at a given concentration in these isolated tissue preparations. It has been proposed that the leptin-mediated inhibition of triacylglycerol synthesis in non-adipose tissue could provide the additional fatty acids for oxidation [12]. However, neither the increased oxidation of intramuscular triacylglycerols nor the partitioning of fatty acids away from esterification and towards oxidation are seen to account quantitatively for the leptin-induced increase in fatty acid oxidation [1,2,4,8]. Finally, while Minokoshi et al. [8] postulated that leptin stimulation of fatty acid oxidation occurred via activation of the AMPK/ACC2/CPTI axis, the role of this pathway has recently been brought into question [13–15]. Taken altogether, it appears that other molecular regulatory mechanisms may be essential for the leptin-stimulated increase in fatty acid oxidation in muscle tissues.

Minokoshi et al. [8] formulated their proposed mechanism of leptin-stimulated fatty acid oxidation before the critical role of fatty acid uptake in regulating fatty acid oxidation had been established. Specifically, in the past decade, a substantial amount of work has shown that fatty acid uptake into muscle tissues is regulated by a protein-mediated mechanism, involving one or more fatty acid transport proteins (cf. [16–18]). Among the known fatty acid transporters, fatty acid translocase (FAT/CD36) is key in taking up fatty acids by muscle and heart (cf. [17]). In both tissues, selected metabolic stimuli, including insulin, muscle contraction, and AMPK activation, induce the translocation of FAT/CD36 from an intracellular depot to the plasma membrane, thereby stimulating the rate of fatty acid uptake [19–26]. The ablation of FAT/CD36 in heart and skeletal muscle largely abrogates (a) insulin-stimulated fatty acid esterification, (b) AMPK-stimulated, and (c) exercise-induced fatty acid oxidation [26–28]. Thus, it appears that FAT/CD36 is particularly important in regulating fatty acid utilization by controlling the rate of fatty acid entry into the myocyte, especially when physiologic signals are present to stimulate fatty esterification or oxidation.

Whether the leptin-induced AMPK activation of fatty acid oxidation is FAT/CD36-dependent is unknown. However, leptin rapidly activates AMPK [8], and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR)-induced AMPK activation in skeletal muscle and cardiac myocytes is involved in stimulating fatty acid oxidation and fatty acid uptake [23,29], by inducing FAT/CD36 translocation [19,25]. Therefore, we hypothesized that leptin, via its activation of AMPK, induces the translocation of FAT/CD36 to the cell surface. This recruitment of FAT/CD36 to the plasma membrane would stimulate fatty acid uptake, thereby providing the necessary additional substrate required for the leptin-stimulated increase in fatty acid oxidation.

We examined these questions in isolated skeletal muscle and in cardiac myocytes, employing a variety of procedures designed to stimulate or inhibit fatty acid uptake and oxidation, as well as modifying AMPK phosphorylation and altering the presence and/or functioning of FAT/CD36. Our studies have revealed a novel mechanism of leptin-induced fatty acid oxidation; namely, this process is critically dependent on the activation of AMPK to induce the translocation of FAT/CD36 to the plasma membrane, thereby stimulating fatty acid uptake. Notably, without up-regulating this FAT/CD36-mediated, fatty acid uptake process, leptin-stimulated phosphorylation of AMPK does not enhance fatty acid oxidation.

Materials and methods

Studies were performed in isolated mouse skeletal muscle and in rat cardiomyocytes, as well as in HL-1 cardiomyocytes. Approval for the experiments was obtained from the committees on animal care at the University of Guelph and Maastricht University.

Materials

Leptin was purchased from Cedarlane Laboratories (Burlington, Ontario). Phloretin and adenine 9-β-D-arabinofuranoside (Ara-A), an AMP analogue, and benzothonium hydroxide, were purchased from Sigma–Aldrich (St Louis, Missouri). [1-^{14}C] palmitate was purchased from Amersham Life Sciences (Little Chalfont, U.K.), and [^{14}C] mannitol was bought from PerkinElmer (Woodbridge, ON). Collagenase type II was obtained from Bioshop Canada, Inc. (Burlington, Ontario). AMPK antibody, phospho-AMPK antibody (Thr-172; Thr-172AMPK; Upstate, Lake Placid, NY), ACC antibody, and phospho-ACC antibody (Ser-79; Ser-79 p-ACC; Cell Signalling Technology, Danvers, MA) were purchased from commercial suppliers. Sulfo-*N*-succinimidyl oleate (SSO) was synthesized in our laboratories. FAT/CD36 was detected using the MO25 antibody [30]. Lipofectamine-2000 was purchased from Invitrogen (San Diego, CA, U.S.A.). Non-coding siRNA and siRNA against the AMPKα2 catalytic subunit (GCACGGUCAAGUUUUGAUUtt) were

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obtained from Ambion Applied Biosystems (Bleiswijk, The Netherlands). The manufacturer specified that the Silencer(r) negative control siRNA was designed to have no significant sequence similarity to mouse, rat, or human transcript sequences. All other reagents were obtained from Sigma–Aldrich (St Louis, MO).

Animals

Mice, wild type (WT), FAT/CD36-KO, and AMPK kinase-dead (AMPK-KD; male 20–24 g), were used for skeletal muscle studies. CD36-KO mice were provided by Dr M. Febbraio (Cleveland Clinic, Cleveland, Ohio, U.S.A.) and AMPK-KD mice [31] by Dr M. Birnbaum (University of Pennsylvania School of Medicine, Philadelphia, PA, U.S.A.). Since cardiac myocytes from mice are difficult to prepare, we used Sprague-Dawley rats (male, 225–250 g) to generate cardiomyocytes. All animals were bred on-site at the University of Guelph. They were maintained at 20°C in a humidity-controlled room on a reverse light–dark (12:12 h) cycle and had access to standard laboratory chow and water *ad libitum*.

Leptin stimulation of fatty acid oxidation in skeletal muscle and cardiomyocytes

Leptin-stimulated palmitate oxidation and its inhibition were determined in isolated extensor digitorum longus (EDL) muscle, and in cardiomyocytes, obtained from anaesthetized WT mice and WT rats, respectively (Somnotol, 60 mg/100 g body weight, i.p.) under three conditions: (i) control, (ii) leptin stimulation, and (iii) leptin in combination with AMPK inhibitors Compound C (50 μM) and Ara-A (2.5 mM) [32–36]. In addition, to discern the roles of the fatty acid transporter FAT/CD36 and AMPK phosphorylation in leptin-stimulated fatty acid oxidation, we also examined the leptin-stimulated palmitate oxidation in isolated EDL muscles of FAT/CD36-KO and AMPK-KD mice, as well as in rat cardiac myocytes using SSO, a well-known blocker of FAT/CD36-mediated fatty acid uptake [21,23,26,28,37,38].

Procedures for determining fatty acid oxidation in skeletal muscle and cardiomyocytes

Skeletal muscles

Fatty acid oxidation was determined either without or with leptin (10 μg/ml, 60 min) in isolated muscles [EDL, 2 ml Medium 199, 30°C (pH 7.4), 4% BSA, 0.5 mM palmitate, [1-^{14}C]-palmitate (1 μCi/vial), 95% O_2, 5% CO_2]. After 60 min, $^{14}CO_2$ was captured from the incubating medium using a benzothonium hydroxide trap. We have described these procedures previously [26,39]. Some muscles were also incubated with AICAR (2 mM) to compare its stimulation of fatty acid oxidation with the leptin-induced increase in fatty acid oxidation.

Cardiomyocytes

Cardiomyocytes were obtained from 20 min perfused rat hearts (Langendorff recirculating mode). Thereafter, palmitate oxidation was determined in cardiac myocytes either without or with leptin (10 μg/ml, 15 min), [Krebs–Henseleit buffer: 2% BSA, 1 mM $CaCl_2$, 95% O_2–5% CO_2, 37°C, pH 7.4, palmitate (100 μM, 1 μCi [1-^{14}C]-palmitate)]. After 15 min, fatty acid oxidation was stopped and $^{14}CO_2$ from the incubation medium was trapped as was done with skeletal muscle. We have described these procedures previously [19,40–42].

Procedures for determining fatty acid uptake in skeletal muscle and cardiomyocytes

Fatty acid uptake was determined in giant sarcolemmal vesicles obtained from leptin-stimulated skeletal muscles of WT, CD36-KO, and AMPK-KD mice, to ascertain whether leptin-stimulated fatty acid uptake via the fatty acid transporter FAT/CD36 involves the activation of AMPK. Similar fatty acid uptake studies were performed in cardiomyocytes, except that we used SSO, the inhibitor of FAT/CD36-mediated fatty acid uptake [28,43,44], to examine whether leptin stimulation of fatty acid uptake occurred via FAT/CD36.

Skeletal muscle

We have routinely used giant sarcolemmal vesicles to examine fatty acid uptake by skeletal muscle [26,44,45]. Because large quantities of muscle are required to prepare these vesicles we infused hindlimb muscles without or with leptin (1 μg/g body weight) [8], via the inferior vena cava in anaesthetized mice, as we have reported recently [45]. To obtain sufficient muscle tissue for preparing a single aliquot of giant sarcolemmal vesicles, it

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was necessary to pool hindlimb muscles from three mice for each independent control and leptin experiment. We performed five to seven such independent experiments for each treatment and for each of the three groups of mice (WT, CD36-KO, and AMPK-KD).

Giant vesicles were prepared from minced muscles treated with collagenase [60 min, 34°C, 140 mM KCl-10 mM MOPS (pH 7.4), type VII collagenase (150 U/ml), and aprotinin (1 mg/ml)]. Subsequently, vesicles were washed and then separated via a density gradient using low-speed centrifugation (60×***g***, 45 min). Subsequently, vesicles were harvested and fatty acid uptake by giant vesicles was determined. We have previously described all these procedures [26,45,46].

Cardiomyocytes

Fatty acid uptake by cardiomyocytes was determined during the last 3 min of a 15-min incubation period as we have described previously [21,23]. To block fatty acid uptake, we used SSO (0.4 mM) as we have previously reported [19,29].

Plasma membrane preparations from skeletal muscle and cardiomyocytes

Selected proteins were determined via standard western blotting in tissue homogenates and on the plasma membrane of muscle and heart.

Skeletal muscle

We used giant sarcolemmal vesicles (see above) to measure FAT/CD36 on the plasma membrane of skeletal muscle, as we have previously reported [18,26,38].

Cardiomyocyte

Plasma membranes were obtained using procedures that we have previously described [19,40–42].

Western blotting

FAT/CD36, AMPKα2, ACC, pAMPK^{Thr172} and pACC^{Ser79} were measured using standard western blotting procedures as we have previously reported [26,45]. Quantification of blots was performed using chemiluminescence (PerkinElmer Life Science, Boston, MA) and ChemiGenius2 Bioimaging (SynGene, Cambridge, U.K.). Equal loading of proteins was determined using caveolin 3, which is not altered by any of the treatments used in the present study.

Effects of reducing AMPK on leptin stimulation of AMPK phosphorylation and plasma membrane FAT/CD36 in HL-1 cardiomyocytes

HL-1 cardiomyocytes [gift from Dr Claycomb (Louisiana State University, New Orleans, U.S.A.)] were cultured and then transfected with non-coding (nc) and AMPK siRNA directed against the AMpkα2 subunit. Thereafter (48 h), we examined the effect of leptin stimulation (10 μg/ml, 30 min) on plasmalemmal FAT/CD36, which was determined using a colorimetric procedure. We have previously reported the siRNA knockdown procedures and the colorimetric determination of plasmalemmal FAT/CD36 [47,48].

Statistics

All data are expressed as mean ± SEM. We used one-way analyses of variance to determine the statistical significance, and a Fisher's least squares *post hoc* test when warranted. Student's t-tests were also used when appropriate. Bonferroni corrections were applied for multiple comparisons. Statistical significance was set at $P \leq 0.05$.

Results

The regulation of leptin-stimulated fatty acid oxidation was examined in skeletal muscle and in cardiomyocytes.

Skeletal muscle

Leptin-stimulated fatty acid oxidation and AMPK phosphorylation in WT muscle

The stimulatory effect of leptin on fatty acid oxidation was examined in isolated EDL muscle of WT mice. Relative to control, leptin increased fatty acid oxidation by +40% ($P < 0.05$; Figure 1A). This increase was

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DOI: 10.1042/BCJ20160804

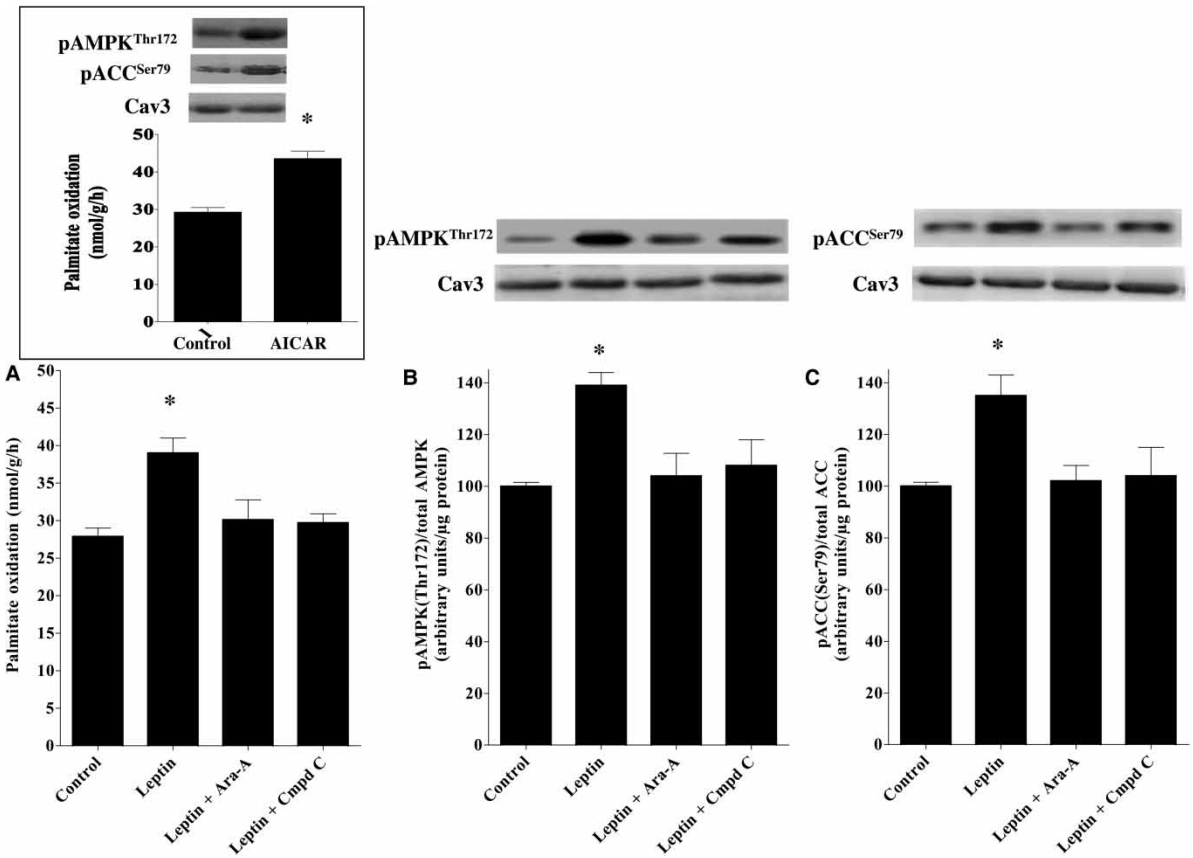

Figure 1. In muscle, leptin stimulates fatty acid oxidation and AMPK phosphorylation.
Effects of leptin, leptin + Ara-A, and leptin + Compound C on palmitate oxidation (**A**), AMPK^{Thr172} phosphorylation (**B**), and ACC^{ser79} phosphorylation (**C**) in isolated skeletal muscle. For comparison purposes, the effects of AICAR on palmitate oxidation are also shown (inset **A**). Data were obtained from isolated skeletal muscles incubated with leptin (**A–C**) or with AICAR (inset, see Materials and Methods for details). pAMPK^{Thr172} and pACC^{Ser79} are shown relative to total AMPK and total ACC, respectively; neither of these totals were altered by any of the treatments used in the present study. Skeletal muscles were incubated with leptin. Data are mean ± SEM. $N = 5–7$. *$P < 0.05$, treatment vs. control.

similar to that induced by AICAR (+49%, $P < 0.05$; Figure 1A, inset), a well-known stimulator of AMPK phosphorylation.

As expected, in WT mice [8], leptin stimulated the phosphorylation of AMPK^{Thr172} ($P < 0.05$; Figure 1B). When leptin-stimulated AMPK^{Thr172} phosphorylation was inhibited by Ara-A or Compound C (Figure 1B), ACC^{Ser79} phosphorylation was blocked (Figure 1C) and leptin-stimulated fatty acid oxidation was inhibited (Figure 1A).

Leptin stimulation of fatty acid oxidation is FAT/CD36-dependent

Given that AICAR-stimulated AMPK activation induces the translocation of FAT/CD36 to the plasma membrane [19,25], we examined (a) whether leptin, via AMPK activation, could induce the translocation of FAT/CD36 to stimulate fatty acid uptake and (b) whether leptin-stimulated, FAT/CD36-mediated fatty acid uptake is required to observe leptin-mediated up-regulation of fatty acid oxidation. For these purposes, studies were performed in WT, FAT/CD36-KO, and AMPK-KD mice, animal models in which the leptin receptor content at the plasma membrane is not altered by leptin (Bonen, unpublished data).

Leptin, AMPK, FAT/CD36, and fatty acid uptake

In WT mice, leptin increased AMPK^{Thr172} phosphorylation by 34% (Figure 2A), while phosphorylation of ACC^{ser79} was also increased (Figure 2B). Leptin also increased plasma membrane FAT/CD36 by 49% (Figure 2C) and fatty acid uptake by 43% (Figure 2D) in WT mice.

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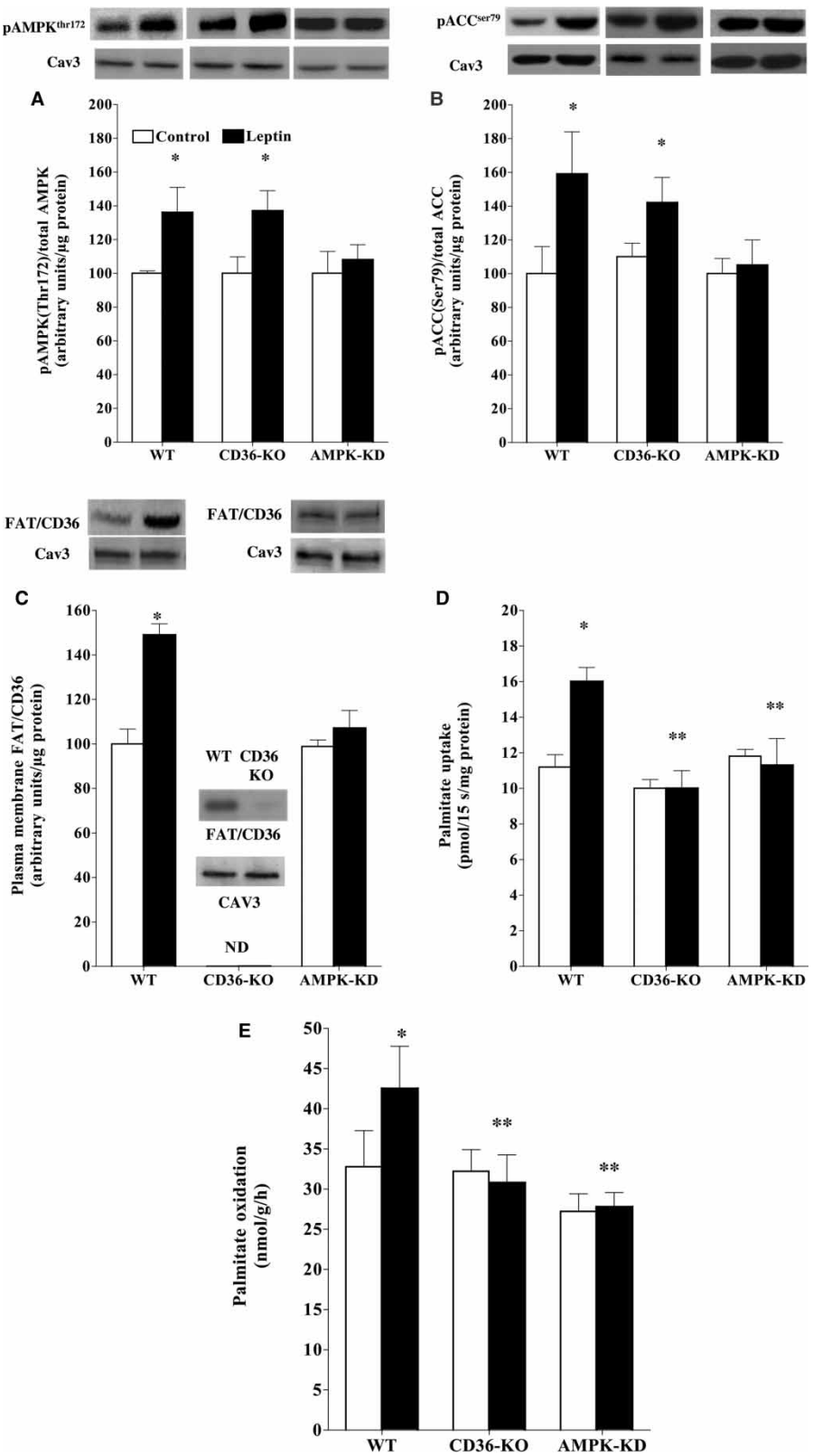

Figure 2. In muscle, leptin activates AMPK and translocates FAT/CD36 to stimulate fatty acid uptake and oxidation.

Part 1 of 2

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Figure 2. In muscle, leptin activates AMPK and translocates FAT/CD36 to stimulate fatty acid uptake and oxidation.

Part 2 of 2

Effects of leptin on AMPK^{Thr172} phosphorylation (**A**), ACC^{ser79} phosphorylation (**B**), plasma membrane FAT/CD36 (**C**), and palmitate uptake (**D**) and oxidation (**E**) in skeletal muscle of WT, FAT/CD36-KO, and AMPK-KD mice. Data in **A–D** were obtained from animals injected with leptin, whereas fatty acid oxidation (**E**) was determined in isolated skeletal muscles incubated with leptin (see Materials and Methods for details). pAMPK^{Thr172} and pACC^{Ser79} are shown relative to total AMPK and total ACC, respectively; neither of these totals were altered by any of the treatments used in the present study. Data are mean ± SEM. $N = 5–7$. $^{*}P < 0.05$, leptin vs. control. $^{**}P < 0.05$, leptin-stimulated FAT/CD36-KO or AMPK-KD vs. leptin-stimulated WT. ND, not determined, as FAT/CD36 is ablated in KO mice.

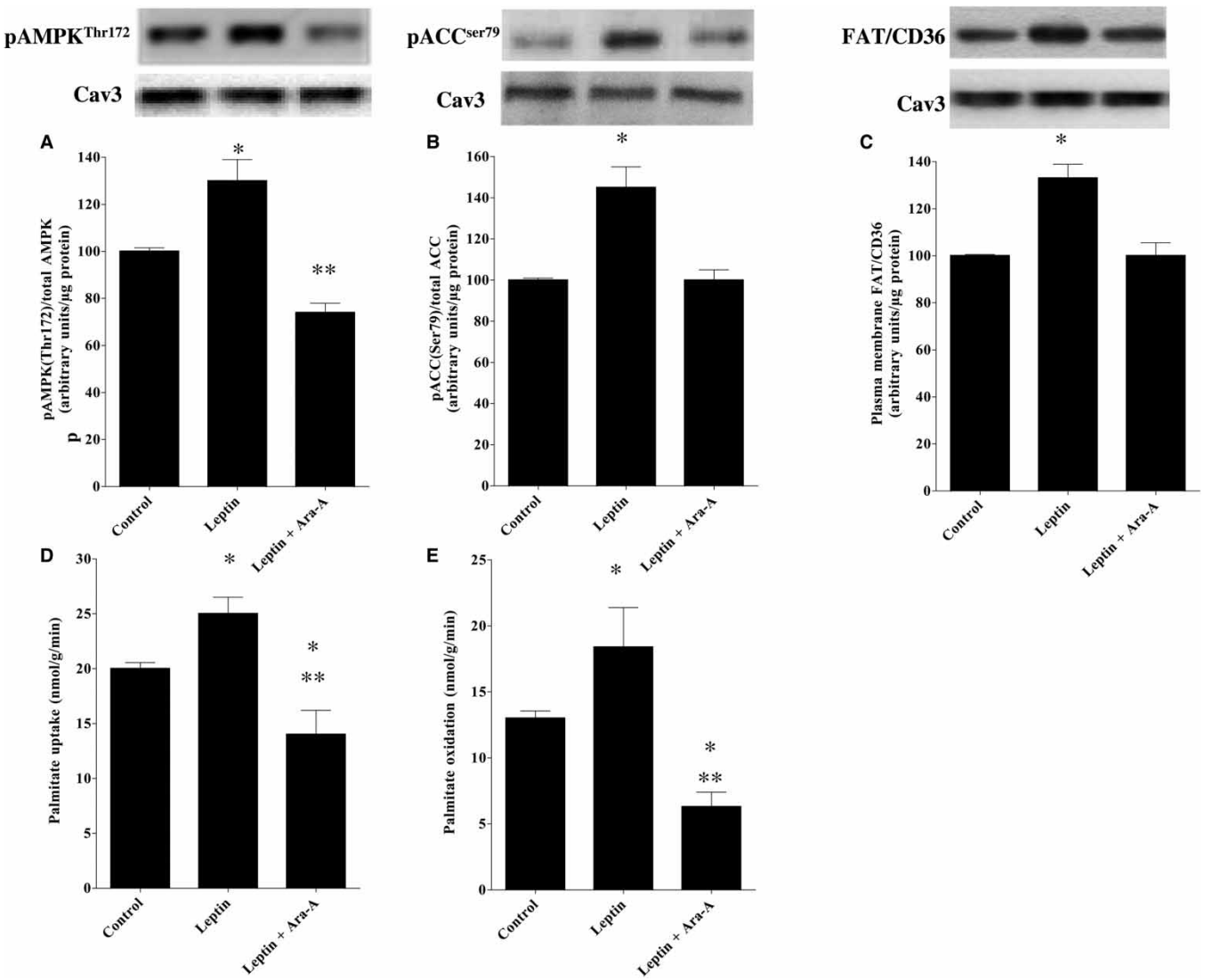

Figure 3. In cardiomyocytes, inhibiting AMPK activation prevents leptin stimulation of fatty acid uptake and oxidation.

Effects of leptin and leptin + Ara-A on AMPK^{Thr172} phosphorylation (**A**), ACC^{Ser79} phosphorylation (**B**), plasma membrane FAT/CD36 (**C**), and palmitate uptake (**D**) and oxidation (**E**) in cardiomyocytes (see Materials and Methods for details). pAMPK^{Thr172} and pACC^{Ser79} are shown relative to total AMPK and total ACC, respectively; neither of these totals were altered by any of the treatments used in the present study. Data are mean ± SEM ($N = 5–8$). $^{*}P < 0.05$, treatment compared with control. $^{**}P < 0.05$, treatment compared with treatment with leptin only.

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As expected, in FAT/CD36-KO mice [26], basal rates of fatty acid uptake were slightly reduced (~10%, Figure 2D). In addition, despite the normal leptin-induced AMPKα2^{Thr172} (+35%) and ACC^{ser79} phosphorylations (+40%) in these animals (Figure 2A,B), leptin failed to stimulate fatty acid uptake in the FAT/CD36-KO mice (Figure 2D).

In AMPK-KD mice, an inactive mutated catalytic subunit of AMPKα2 replaces the endogenous form [31]. In these mice, leptin did not enhance AMPK^{Thr172} phosphorylation (Figure 2A) nor ACC^{ser79} phosphorylation (Figure 2B). In addition, in the AMPK-KD mice, leptin failed to induce the translocation of FAT/CD36 (Figure 2C) and failed to increase the rate of fatty acid uptake (Figure 2D).

FAT/CD36 is central to leptin-stimulated fatty acid oxidation

Leptin stimulation of fatty acid oxidation observed in WT mice did not occur when FAT/CD36 was ablated (Figure 2E), despite comparable increases in leptin-stimulated AMPK^{Thr172} and ACC^{Ser79} phosphorylation in

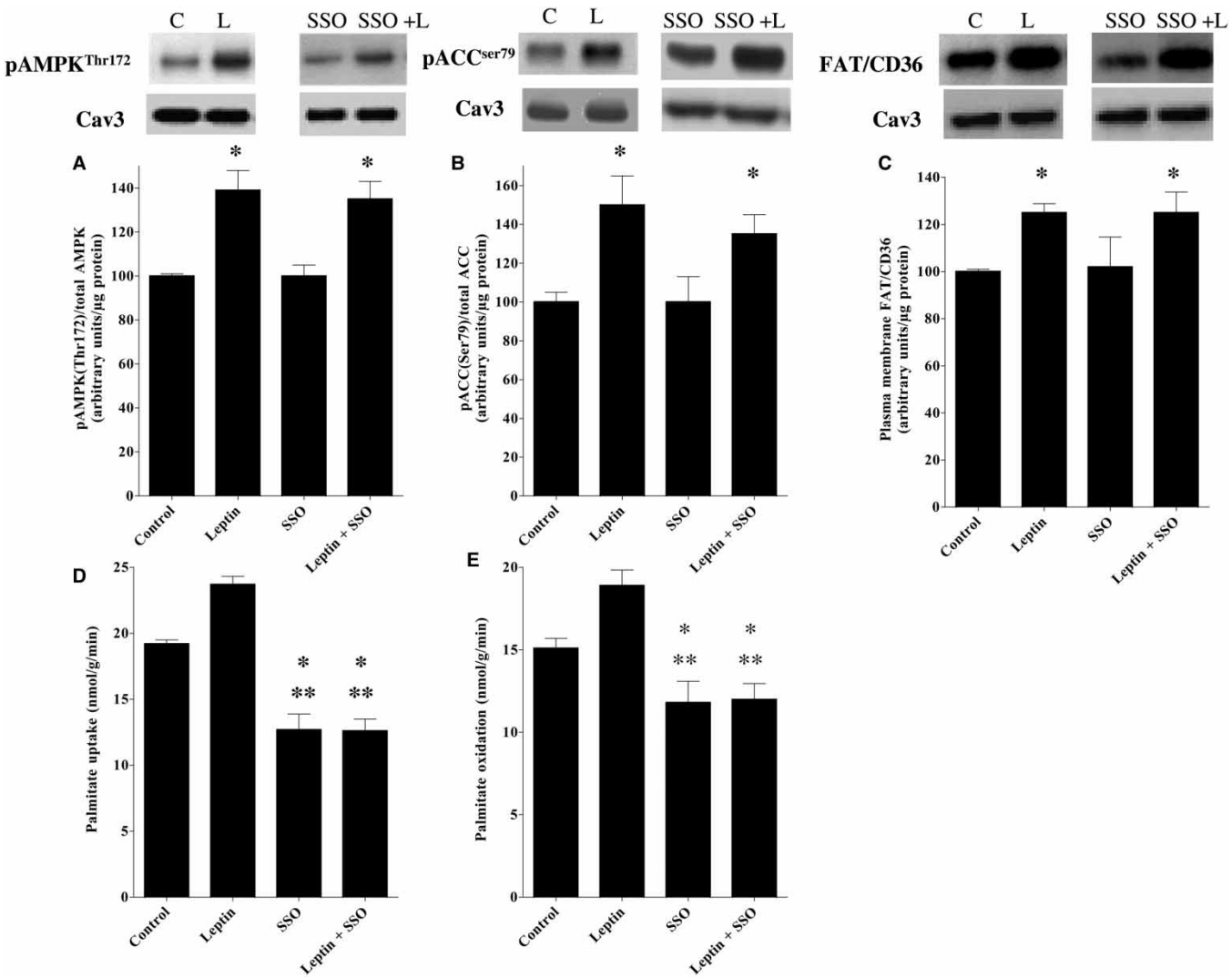

Figure 4. In cardiomyocytes, blocking plasmalemmal FAT/CD36 inhibits leptin stimulation of fatty acid oxidation.

Effects of leptin, SSO, and leptin + SSO on AMPK^{Thr172} phosphorylation (**A**), ACC^{Ser79} phosphorylation (**B**), plasma membrane FAT/CD36 (**C**), and palmitate uptake (**D**) and oxidation (**E**) in cardiomyocytes (see Materials and Methods for details). pAMPK^{Thr172} and pACC^{ser79} are shown relative to total AMPK and total ACC, respectively; neither of these totals were altered by any of the treatments used in the present study. Data are mean ± SEM ($N = 5–8$). *$P < 0.05$, treatment compared with control. **$P < 0.05$, treatment compared with treatment with leptin only.

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WT and FAT/CD36-KO mice (Figure 2A,B). Similarly, leptin also failed to stimulate fatty acid oxidation in AMPK-KD mice (Figure 2E), animals in which leptin was unable to increase plasmalemmal FAT/CD36 and to phosphorylate AMPK^{Thr172} and it downstream substrate ACC^{ser79} (Figure 2A,B).

Cardiac myocytes

In cardiac muscle, energy production relies mainly on fatty acid oxidation. Therefore, we examined whether leptin stimulation of fatty acid oxidation in cardiac myocytes was also dependent on leptin-induced increases in plasma membrane FAT/CD36 and fatty acid uptake. These studies showed that, just as in skeletal muscle, leptin stimulated AMPK^{Thr172} phosphorylation (Figure 3A) and ACC^{ser79} phosphorylation (Figure 3B), and increased plasma membrane FAT/CD36 (Figure 3C), palmitate uptake (Figure 3D), and palmitate oxidation (Figure 3E). When AMPK activation was blocked with Ara-A, all the leptin-stimulated processes were inhibited (Figure 3B–E).

Leptin-stimulated fatty acid oxidation is FAT/CD36-dependent

To discern the role of the FAT/CD36 and fatty acid uptake in the leptin stimulation of fatty acid oxidation, further studies in cardiomyocytes were performed to examine leptin stimulation of AMPK phosphorylation and fatty acid uptake in the presence of SSO, a specific inhibitor of plasmalemmal FAT/CD36 [43,44,49–51]. These studies showed that, under basal conditions, SSO on its own did not alter AMPK^{Thr172} or ACC^{ser79} phosphorylations (Figure 4A,B) or levels of plasma membrane FAT/CD36 (Figure 4C). Similarly, SSO did not

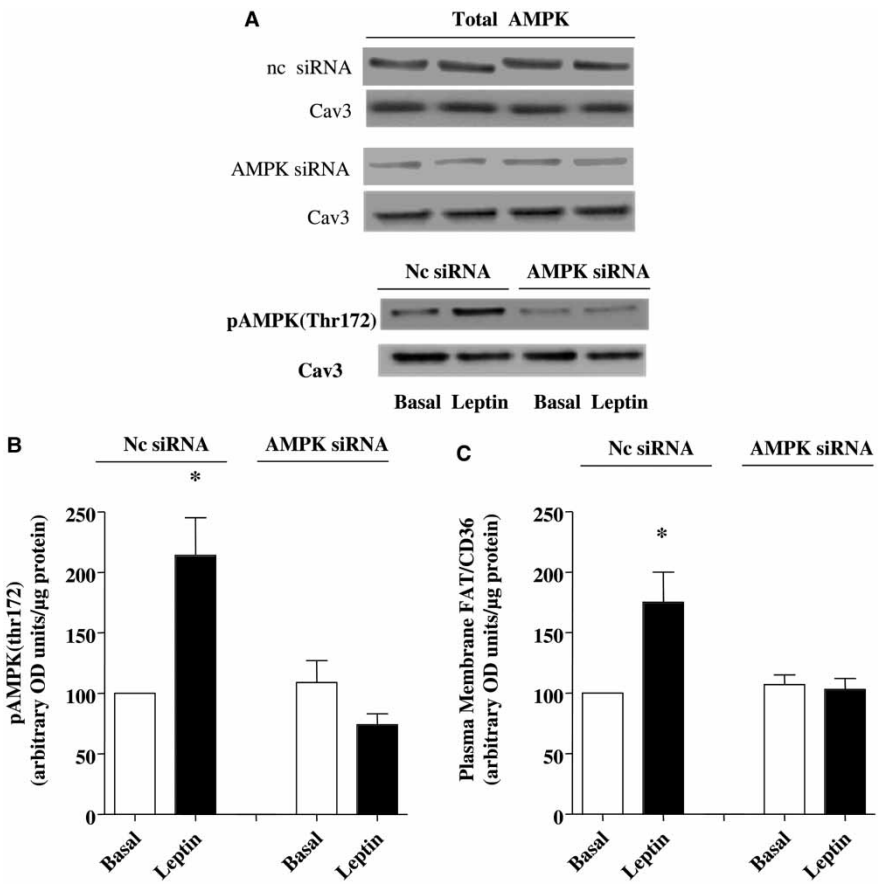

Figure 5. In HL-1 cardiomyocytes, reducing AMPK inhibits leptin-stimulated AMPK phosphorylation and FAT/CD36 translocation.

Effects of AMPK siRNA on AMPK (**A**), leptin-stimulated AMPK^{Thr172} phosphorylation (**B**), and plasma membrane FAT/CD36 (**C**) in HL-1 cells. HL-1 cells were incubated with leptin (see Materials and Methods for details). Data are mean ± SEM ($N = 4$). Nc siRNA, non-coding siRNA. *$P < 0.05$, leptin compared with basal.

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impair leptin-stimulated AMPK^{Thr172} or ACC^{ser79} phosphorylation (Figure 4A,B), nor the leptin-stimulated increase in plasma membrane FAT/CD36 (Figure 4C). However, SSO did inhibit palmitate uptake, either under basal conditions or during stimulation with leptin (Figure 4D. Concomitantly, fatty acid oxidation (Figure 4E) was also inhibited when fatty acid uptake (Figure 4D) was impaired. In knockdown experiments, using AMPK siRNA in HL-1 cells, a cardiac cell line (Figure 5A), leptin-stimulated AMPK^{Thr172} phosphorylation (Figure 5B), and FAT/CD36 translocation (Figure 5C) were impaired.

Discussion

We have identified a novel regulatory system that is part of the known leptin-induced stimulation of fatty acid oxidation in muscle tissues. This effect of leptin was found to be completely reliant on increasing FAT/CD36-mediated fatty acid uptake across the plasma membrane. Specifically, we have shown that (a) leptin-stimulated phosphorylation of AMPK is necessary to induce the translocation of FAT/CD36 to the plasma membrane, thereby increasing fatty acid uptake and (b) that when FAT/CD36 is ablated, or is blocked by SSO, leptin-stimulated activation of AMPK fails to up-regulate fatty acid oxidation. Thus, the present study has revealed a key leptin-mediated mechanism for stimulating fatty acid oxidation, a process that is FAT/CD36-dependent.

It is well known that leptin stimulates the rate of fatty acid oxidation in many tissues, including skeletal muscle and the heart [1,2,4,5,8]. Whether leptin stimulation of fatty acid oxidation necessarily involves the AMPK/ACC2/CPT-I axis, as originally proposed [8], has been questioned [13–15]. Moreover, in some studies [1,2], but not in all [4,8], leptin-stimulated fatty acid oxidation has been attributed, in part, to reduced rates of fatty acid esterification. Such reciprocal changes in fatty acid esterification and oxidation, when they occur, may be orchestrated by leptin-mediated increases in AMPK, since its activation stimulates both ACC phosphorylation and *sn*-glycerol-3-phosphate phosphorylation [52], thereby simultaneously increasing fatty acid oxidation and decreasing their esterification [52]. However, many studies have shown that these reciprocal changes cannot account for the leptin stimulation of fatty acid oxidation [1,2,4,8]. This prompted our search for an additional leptin-stimulated mechanism, one that provided additional fatty acids to the mitochondria. Since muscle contraction [23,26,38], oligomycin, and AICAR [19,23] each stimulate AMPK phosphorylation, and induce the translocation of FAT/CD36 from an intracellular depot to the plasma membrane, as well as stimulating fatty acid uptake and fatty acid oxidation, we hypothesized that leptin activation of AMPK [8] might well be critical for inducing FAT/CD36 translocation, thereby providing a means to quickly increase fatty acid uptake as a necessary precondition for the rapid leptin-mediated increase in the rate of fatty acid oxidation.

In the present study, we provide evidence for the role of the FAT/CD36-mediated fatty acid uptake in leptin-stimulated fatty acid oxidation, based on observations that fatty acid oxidation was entirely blocked, when FAT/CD36 was either ablated or when plasmalemmal FAT/CD36 was blocked with SSO, a specific inhibitor of FAT/CD36 [43,44,49–51]. In previous studies, we have shown that SSO blocked FAT/CD36-mediated cellular fatty acid uptake in heart and skeletal muscle from WT rodents, whereas in FAT/CD36-null mice SSO failed to lower further the reduced fatty acid uptake [21,23,26,28,37,38]. Moreover, SSO is known to be cell impermeant, making it unlikely that this inhibitor exerted any intracellular metabolic effects. Accordingly, SSO did not prevent the leptin-induced increase in plasmalemmal FAT/CD36, which reflects its translocation from intracellular depots to the plasma membrane [17,20,38,53]. Taken altogether, our present experiments indicate that by impairing leptin-stimulated FAT/CD36-mediated cellular fatty acid uptake, leptin-stimulated fatty acid oxidation is also impaired.

Our study provides evidence that while leptin induces AMPK activation, this is required to stimulate fatty acid uptake. Specifically, a leptin-induced 40% increase in AMPK^{Thr172} phosphorylation was sufficient to observe substantial metabolic effects of leptin, namely, increased rates of both fatty acid uptake and oxidation in cardiac myocytes and skeletal muscle. However, our work has shown for the first time that the translocation of FAT/CD36 to the plasma membrane was essential to observe both of these leptin-induced metabolic effects. When AMPK^{Thr172} phosporylation was impaired, the metabolic effects normally provoked by leptin were lost, namely the translocation of FAT/CD36, the phosphorylation of ACC^{ser79}, and the increases in fatty acid uptake and oxidation. In addition, when FAT/CD36 was either ablated or was blocked with SSO at the plasma membrane, leptin-stimulated fatty acid uptake was impaired as was the increase in fatty acid oxidation, despite a leptin-induced increase in AMPK^{Thr172} phosporylation. Taken altogether, our experiments indicate that rapid leptin-induced AMPK^{Thr172} phosphorylation is central to not only activating fatty acid oxidation (present study and [1,2,4,8]) but also stimulating fatty acid uptake by inducing the translocation of FAT/CD36 from an intracellular compartment to the plasma membrane (present study).

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DOI: 10.1042/BCJ20160804

AMPK can be phosphorylated at Thr172 on both its α1 and α2 subunits. However, leptin most probably phosphorylates AMPKα2 for many reasons. In contrast with most tissues, in which AMPKα1 accounts for 60–90% of AMPK activity, this is not the case in muscle and heart. In these tissues, AMPKα2 is dominant, accounting for 70–80% of total AMPK activity [54,55]. Presumably, this suggests that the AMPK^{thr172} phosphorylation measured in the present study largely reflects the phosphorylation of AMPKα2^{thr172}. This is further supported by several other lines of evidence. Specifically, it was shown by Minokoshi et al. [8] that leptin selectively stimulates the phosphorylation of AMPKα2^{Thr172} in skeletal muscle. In addition, in the AMPK-KD mice used in our present studies, AMPKα2 is kinase-dead [31], and in this AMPKα2 kinase-dead model, we observed that leptin failed to alter AMPKα^{thr172} phosphorylation. Taken altogether, it appears that the metabolic action of leptin with respect to FAT/CD36-mediated fatty acid uptake and oxidation probably occurs via the activation of AMPKα2^{thr172}.

Our experimental observations combined make considerable sense, as leptin-stimulated AMPK^{Thr172} activation is seen to provide a mechanism to stimulate fatty acid uptake into the cell, via the increase in plasmalemmal FAT/CD36, thereby increasing the intracellular fatty acid supply for mitochondrial oxidation. Based on our findings, we present a revised scheme by which leptin stimulates fatty acid oxidation; this process necessarily requires AMPK activation to stimulate FAT/CD36-mediated cellular fatty acid uptake (Figure 6).

Caveats

We are aware that Ara-A, Compound C, and SSO can have non-specific metabolic effects. However, with respect to the parameters investigated in the present study, the inhibition of fatty acid uptake by SSO and the inhibition of AMPK^{Thr172} phosporylation, by Ara-A and Compound C, were congruent with those observed in FAT/CD36-KO and AMPK-KD animals, respectively.

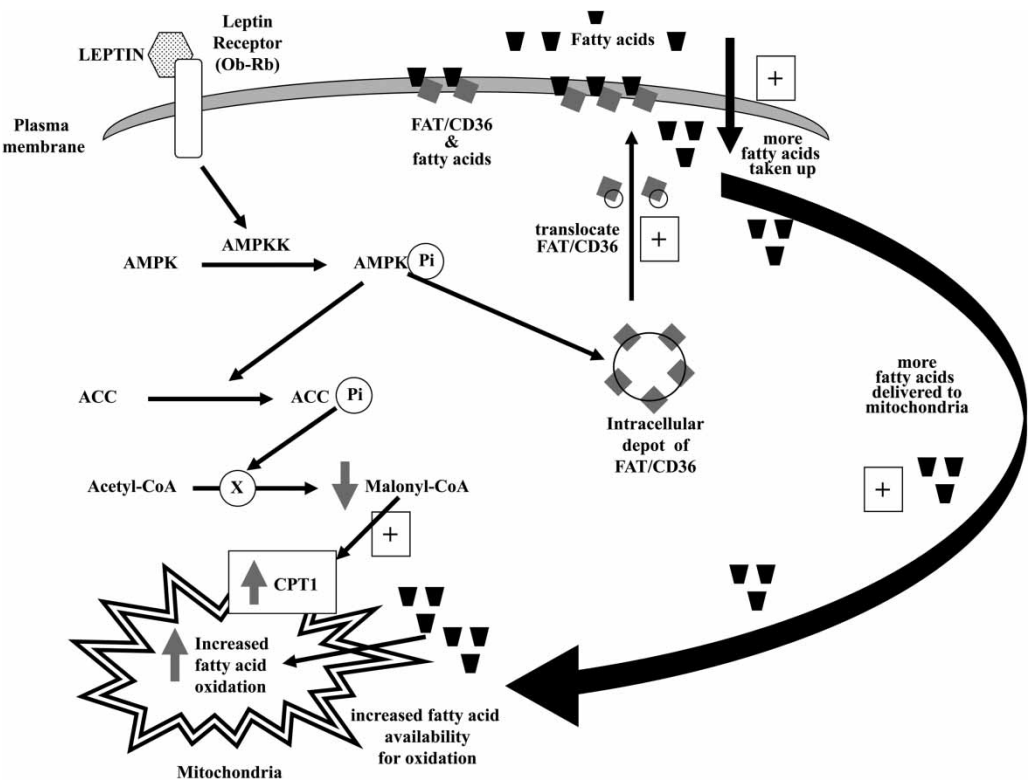

Figure 6. Schematic representation of leptin-induced stimulation of fatty acid utilization showing that leptin phosphorylates AMPK, which phosphorylates ACC to increase fatty acid oxidation [56]. However, AMPK activation also induces the translocation of FAT/CD36 to the plasma membrane, which in turn up-regulates the uptake of fatty acids, which can then be delivered to mitochondria where they are oxidized. Importantly, without the leptin-stimulated FAT/CD36-mediated influx of fatty acids, leptin fails to up-regulate fatty acid oxidation even when AMPK is activated.

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DOI: 10.1042/BCJ20160804

We recognize that other metabolic stimuli besides leptin can up-regulate fatty acid oxidation in an AMPK-independent manner, as has been shown in contracting skeletal muscle [25] and in working hearts in which leptin failed to alter AMPK activity [4]. Nevertheless, in quiescent cardiac myocytes and resting skeletal muscle, leptin phosphorylation of $AMPK^{Thr172}$ is key to up-regulating fatty acid uptake and oxidation.

Summary

We have shown that leptin-stimulated fatty acid oxidation is critically dependent on the leptin-stimulated phosphorylation of AMPK, which induces the translocation of FAT/CD36 from an intracellular depot to the plasma membrane. This leptin-induced translocation of FAT/CD36 and the subsequent increase in fatty acid uptake is crucial, since blocking of plasmalemmal FAT/CD36 abrogated the stimulatory effects of leptin on fatty acid uptake and subsequently fatty acid oxidation, even when AMPK was phosphorylated normally.

Abbreviations

ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; AMPK, AMP-activated protein kinase; AMPK-KD, AMPK kinase-dead; Ara-A, adenine 9-β-D-arabinofuranoside; CPTI, carnitinepalmitoyltransferase 1; EDL, extensor digitorum longus; FAT/CD36, fatty acid translocase; KO, knock-out; nc, non-coding; SSO, sulfo-*N*-succinimidyloleate; WT, wild type.

Author Contribution

A.B. conceived, designed, and supervised the study, and analyzed the data. I.M. and A.B. wrote the manuscript. I.M., A.C., E.D., M.N., S.S.J., J.T.M., and J.J.F.P.L. performed experiments, interpreted data, and contributed to revising the manuscript. A.C., J.F.C.G., and J.J.F.P.L. contributed intellectual expertise, interpreted data, and reviewed and edited the manuscript. All authors contributed to reviewing and editing the manuscript, and approved its final version.

Funding

These studies were supported in part by grants from the Heart and Stroke Foundation of Ontario, the Natural Sciences and Engineering Research Council of Canada, the Canadian Institute of Health Research, the Transnational University Limburg, the Netherlands Organization of Scientific Research [NWO-ZonMw VENI grant no. 916.14.050 to M.N.], Grant N/ST/ZB16/001/1118, MUB, Poland, and the Canada Research Chair program. A.B. was the Canada Research Chair in Metabolism and Health.

Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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