Phosphatidylinositol 3-kinase-dependent insulin regulation of long-chain fatty acid (LCFA) metabolism in L6 muscle cells: involvement of atypical protein kinase C- ζ in LCFA uptake but not oxidation

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Abstract

Insulin is important in the regulation of muscle metabolism. However, its role in the regulation of muscle long-chain fatty acid (LCFA) metabolism, independent of glucose, is not clear. To determine whether insulin regulates LCFA metabolism independent of glucose and if so, via which signaling pathway, L6 myotubes were incubated, in the presence or absence of insulin (100 nM) and with either an inhibitor of phosphatidylinositol 3-kinase (PI3K) (wortmannin (W), 50 nM), protein kinase B (PKB)/Akt (A, 10 μ M), or atypical protein kinase C- ζ (aPKC- ζ) (mP, 100 μ M). LCFA kinetic parameters were measured via incubation with [1-¹⁴C]palmitate. Basal LCFA uptake was found to increase linearly with time

(1–60 min) and concentration (50–750 μ M). LCFA uptake increased in the presence of insulin and was maximum at 10 nM ($P < 0.05$). Wortmannin prevented the insulin-induced increase in LCFA uptake and decrease in LCFA oxidation. While mP abolished the insulin-induced increase in LCFA uptake, it did not prevent the insulin-induced decrease in LCFA oxidation. None of the variables were affected by Akt inhibition. These results suggest a direct effect of insulin on LCFA metabolism in muscle cells, and that downstream of PI3K, aPKC- ζ , but not PKB/Akt mediates the effects of insulin on LCFA uptake but not oxidation.

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Introduction

It is well known that insulin affects skeletal muscle metabolism by increasing glucose uptake via GLUT-4 translocation from sarcolemmal vesicles to the plasma membrane (PM) and glycogen synthesis via activation of glycogen synthase (Salteil & Kahn 2001). More recently, in muscle perfused or incubated with glucose, insulin has also been shown to decrease long-chain fatty acid (LCFA) oxidation, increase triacylglycerol (TG) synthesis, and increase LCFA uptake via translocation of the LCFA transporter, fatty acid translocase (FAT/CD36), from an intracellular compartment to the PM (Dyck *et al.* 2001, Luiken *et al.* 2002). However, the effects of insulin on muscle LCFA metabolism, independent of its stimulatory effects on glucose uptake and glycogen synthesis, have not been clearly defined.

It is well accepted that the effects of insulin on carbohydrate metabolism in skeletal muscle occur in large part via activation of phosphatidylinositol 3-kinase (PI3K) and other downstream molecules such as protein kinase B/Akt (PKB/Akt) and atypical protein kinase C- ζ (aPKC- ζ) (Tsakiridis *et al.* 1995, Yeh *et al.* 1995, Bandyopadhyay *et al.* 1997, Gonzalez & Sanchez 2006). While it has been shown that PI3K is also part

of the insulin-induced signaling cascade involved in the regulation of muscle LCFA metabolism (Dyck *et al.* 2001, Luiken *et al.* 2002), the signaling molecules located downstream of PI3K and responsible for the insulin-induced changes in LCFA metabolism are not known. Recently, insulin-mediated PKB/Akt activation was found to be involved in the regulation of lipolysis via changes in the activation state of hormone-sensitive lipase (HSL) in adipose tissue (Gonzalez & Sanchez 2006). Indeed, PKB/Akt was found to be the kinase responsible for the phosphorylation and activation of phosphodiesterase 3B and for the concomitant decrease in cAMP levels, HSL activity, and TG breakdown (Degerman *et al.* 1998). Since it has been suggested that PKB/Akt may be involved as a signaling molecule in the insulin-induced pathway that regulates the insulin-mediated decrease in lipolysis in adipocytes, we aimed to determine whether PKB/Akt might also be involved in the regulation of LCFA uptake and oxidation by insulin in muscle cells.

Likewise, aPKC- ζ is regarded as an important signaling molecule downstream of PI3K in the insulin signaling cascade in skeletal muscle (Koivisto *et al.* 1991, Bandyopadhyay *et al.* 1997, Sajan *et al.* 2006). This view is supported by evidence showing that impaired activation of aPKC- ζ in muscle of

diabetic subjects (Beeson *et al.* 2005) and in myotubes of obese glucose-intolerant subjects (Vollenweider *et al.* 2002, Watt *et al.* 2006) is associated with peripheral insulin resistance as measured by a decrease in insulin-sensitive glucose uptake and disposal. Because abnormalities in lipid metabolism have been directly linked to the development of insulin resistance (Kelley & Mandarino 2000), it is imperative to determine the signaling molecules involved in the insulin-mediated regulation of LCFA metabolism in muscle. Combined with evidence showing that aPKC- ζ mediates, at least in part, the effects of insulin on lipid synthesis in liver (Taniguchi *et al.* 2006), these results suggested that insulin might mediate its effects on LCFA metabolism via the aPKC- ζ pathway. Thus, we aimed to determine whether the insulin-mediated activation of aPKC- ζ is part of the insulin-induced signaling cascade that regulates LCFA uptake and oxidation in muscle cells.

Therefore, the primary purpose of this study was to determine whether insulin regulates LCFA metabolism, independent of glucose, in muscle cells and to gather evidence for the involvement of PI3K-PKB/Akt and/or PI3K-aPKC- ζ signaling in this regulation. L6 myotubes were employed in this study because it is widely recognized that cell culture systems offer unique advantages over intact muscle preparations including cell homogeneity and even accessibility to substrates and hormones. Furthermore, L6 myotubes are commonly used for muscle metabolism studies because they contain insulin receptors with its complement of signaling cascades, glucose, LCFA, and lactate transporter proteins, as well as metabolic enzymes and last but not least allow for an accurate measurement of LCFA uptake (Shainberg *et al.* 1971, Beguinot *et al.* 1986, Mitumoto & Klip 1992, Tsakiridis *et al.* 1995, Somwar *et al.* 1998, Hashimoto *et al.* 2006).

Materials and Methods

Materials

α -Minimal essential medium (MEM), fetal calf serum, and trypsin-EDTA were purchased from Cell Culture Facility (University of Southern California, Los Angeles, CA, USA). Antimycotic-antibiotic solution and wortmannin (W) were purchased from Sigma-Aldrich Ltd. The Akt inhibitor (A), 1L-6-hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate, and the aPKC- ζ inhibitor, myristoylated protein kinase C- ζ pseudosubstrate (mP), were purchased from CalBiochem (San Diego, CA, USA). Antibodies for phosphorylated acetyl-CoA carboxylase (ACC) (Ser⁷⁹), total ACC, phosphorylated phosphoinositol-dependent kinase-1 (PDK-1) (Ser²⁴¹), phosphorylated Akt (Ser⁴⁷³), phosphorylated PKC- ζ / λ (Thr^{410/403}), and anti-PKC- ζ were purchased from Cell Signaling Technologies (Beverly, MA, USA). Anti-Akt1/2 and GAPDH were purchased from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA, USA) and CD36 was purchased from

Cascade Bioscience (Winchester, MA, USA) respectively. Purified polyclonal antibodies to anti-FABP_{PM} were produced as described previously (Turcotte *et al.* 2002a,b), and have been used routinely by others and us (Kiens *et al.* 1997, Tucker & Turcotte 2002, Turcotte *et al.* 2002a,b). Horseradish peroxidase (HRP) goat anti-rabbit secondary antibody and reagents (Super Signal West Pico) and film (CL-Xposure) for enhanced chemiluminescence (ECL) were purchased from Pierce (Rockford, IL, USA). Pork insulin was purchased from the University of Southern California Pharmacy (Los Angeles, CA, USA). Wako NEFA C test kit was purchased from Wako Chemicals (Richmond, VA, USA). [1-¹⁴C]palmitate was purchased from MP Biomedical (Irvine, CA, USA) and [1-¹⁴C]oleate, [1-¹⁴C]acetic acid, and [³H]-2-deoxyglucose (2-DG) were purchased from Perkin-Elmer (Wellesley, MA, USA). Bradford reagent and gel electrophoresis supplies were from Bio-Rad.

L6 cell culture

L6 myotubes were cultured in α -MEM+ containing 2% fetal calf serum and 1% antimycotic-antibiotic solution in a humidified incubator at 37 °C/5% CO₂. Cells were grown in 75 cm² sterile culture flasks, sub-cultured at 60–80% confluence, split at a ratio of 1:10 using trypsin, and sub-cultured in 6-well dishes. By day 3, the cells were 100% confluent and spontaneously differentiated into myotubes as verified by inverted phase contrast microscopy. For experiments, L6 myotubes that were 10 days post-confluent were used.

Cell treatments

Prior to all experimental treatments, cells were pre-incubated in serum-free medium for 90 min (except for glucose uptake, see below), followed by incubation in Krebs-Ringer-HEPES buffer (KRB) (1.47 mM K₂HPO₄, 140 mM NaCl, 1.7 mM KCl, 0.9 mM CaCl₂, 0.9 mM MgSO₄, 20 mM HEPES) for 30 min. The cells were then pre-exposed either to the inhibitors (A, 10 μ M; mP, 100 μ M; W, 50 nM) or vehicle (KRB) for 30 min prior to exposure to either insulin (100 nM) or vehicle (KRB) for 15 min and [1-¹⁴C]palmitate bound to albumin (100 μ M) for 30 min. All incubations took place at 37 °C (95% O₂/5% CO₂). The time and dose-response curves shown in Fig. 2A and B were used to determine the duration of incubation and concentrations of insulin to be used for the experimental conditions. To characterize the insulin-induced signaling pathways involved in the regulation of LCFA metabolism, the effects of PI3K, PKB/Akt, and aPKC- ζ inhibition on insulin-mediated palmitate uptake and oxidation were determined. For each inhibitor, a dose-response curve was used to determine the optimal concentration for the measurement of palmitate uptake (Fig. 3A–C). For these experiments, L6 myotubes were pre-incubated in the presence or absence of W (0–100 nM), A (0–50 μ M), or mP (0–100 μ M) or vehicle (KRB) for 30 min. This was followed by exposure to insulin (100 nM) or vehicle (KRB) for 15 min and [1-¹⁴C]palmitate bound to albumin (100 μ M) for 30 min. To eliminate the

stimulatory effects of insulin on glucose uptake and glucose oxidation and the accompanying indirect effects on LCFA oxidation (Sidossis & Wolfe 1996, Saha *et al.* 1997, Ueki *et al.* 1998), we eliminated glucose from the medium during incubations with metabolic inhibitors and/or insulin and during incubation with the experimental medium for the measurement of palmitate kinetics.

Palmitate uptake

Following inhibitor treatment and/or insulin exposure, the incubation medium was replaced with 1.0 ml experimental medium (100 μ M albumin-bound palmitate, 1:1) containing [$1\text{-}^{14}\text{C}$]palmitate (5 μ Ci/ml) for 30 min, an incubation duration that has been shown to have no effect on insulin sensitivity in L6 cells (Sinha *et al.* 2004). Incubations were terminated by removing the media, and this was used to assay for ^{14}C -labeled oxidation products (see below). Wells were washed twice with KRB and the cells were lysed with 0.05% SDS at room temperature for 30 min on a shaking orbitron. Duplicate 350 μ l aliquots of lysate were taken and mixed with scintillation fluid (BudgetSolve; Research Product International, Mount Prospect, IL, USA) and counted in a Tri-Carb liquid scintillation analyzer (model 2100TR; Packard, Meriden, CT, USA). Duplicate aliquots (10 μ l) of the same lysate were taken for protein determination using the Bradford method. Parallel studies were performed, in duplicate, using unlabelled palmitate bound to albumin (1:1) (100 μ M) to determine the activation state of signaling molecules by western blotting.

Palmitate oxidation

To assay oxidation products, semi-dry filter paper (Whatman, Florham Park, NJ, USA) was glued to caps of vials and was saturated with 100 μ l ethanolamine, as described previously (Turcotte *et al.* 1999). Adaptations to the previously described protocol are as follows. Following the 30-min incubation with [$1\text{-}^{14}\text{C}$]palmitate, duplicate aliquots of experimental media (450 μ l) were transferred to plastic scintillation vials containing 500 μ l of 70% (v/v) perchloric acid and the caps were immediately placed on vials to trap released $^{14}\text{CO}_2$. After 24 h, the caps were transferred to new vials containing ethylene glycol monomethyl ether to remove the filter paper. Toluene cocktail (1 M toluene, 22 mM 2,5-diphenyloxazole (PPO), 0.82 mM 1,4-bis (4-methyl-5-phenyl-oxazol-2-benzene) (POPOP)) was added and $^{14}\text{CO}_2$ was counted via scintillation counting as described above.

To verify that our chosen incubation time of 30 min would not result in deleterious effects on insulin signaling and to demonstrate that there is no difference between the use of palmitate or oleate under our experimental conditions, additional cells were treated as described above and incubated with or without insulin (100 nM) for 15 min followed by exposure to [$1\text{-}^{14}\text{C}$]oleate for 30 min ($N=12$ per condition). Oleate uptake and oxidation were measured as described

above, and these values were compared with those obtained with [$1\text{-}^{14}\text{C}$]palmitate.

To correct for carbon loss, additional experiments were conducted to determine the acetate correction factor under our experimental conditions (Sidossis *et al.* 1995, Tucker & Turcotte 2002). Thus, in subsamples of cells ($n=3$ for each condition), the cells were treated as above except that 5 μ Ci [$1\text{-}^{14}\text{C}$]acetate were added to the incubation medium rather than [$1\text{-}^{14}\text{C}$]palmitate. The samples were taken as described above and analyzed for [^{14}C]acetate and $^{14}\text{CO}_2$ radioactivities.

Glucose uptake

Cells were pre-incubated in serum-free medium for 5 h after which cells were washed twice with warm KRB buffer (Klip *et al.* 1984, Cross *et al.* 1994). Myotubes were either pre-exposed to W (50 nM) or vehicle (KRB) for 30 min, and then incubated in the presence or absence of insulin (100 nM) and/or W (50 nM) or vehicle (KRB) for 15 min. Following exposure to insulin, insulin+W or vehicle, the cells were incubated in 200 μ M 2-DG (0.5 μ Ci/ml) for 5 min. Incubations were terminated via removal of the media. Individual wells were washed twice with KRB after which cells were lysed with 0.05% SDS at room temperature for 30 min on a shaking orbitron. Duplicate 350 μ l aliquots of lysate were taken for scintillation counting. Duplicate aliquots (10 μ l) of the same lysate were taken for protein determination using the Bradford method.

Western blotting

After the experimental treatment, cells were washed with ice-cold KRB. Lysis buffer was added (20 mM Tris, 1% NP-40, 137 mM NaCl, 1 mM CaCl_2 , 1 mM MgCl_2 , 10% (v/v) glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 mM Na_3VO_4) and the cells were scraped to one side of the well. The lysates were transferred to microcentrifuge tubes, gently pelleted for 30 min at 4 $^\circ\text{C}$, and then spun at maximal speed of 15 min. The supernatants were collected and aliquots (10 μ l) assayed for protein content (Bradford method). Cell supernatants were subjected to 7.5% SDS-PAGE. The separated proteins were transferred onto Immobilon-P polyvinylidene difluoride (PVDF) membranes and blocked with 1% BSA in Tween-TBS (TTBS; 500 mM NaCl, 20 mM Tris, 0.05% Tween 20 (pH 7.5)) for 1 h (23 $^\circ\text{C}$) on a shaking orbitron, rinsed three times with TTBS, and incubated overnight with antibodies to either phospho-ACC (Ser⁷⁹) (1:1000), phosphorylated PDK-1 (Ser²⁴¹) (1:1000), phospho-Akt (Ser⁴⁷³) (1:1000), Akt1/2 (1:1000), phospho-aPKC- ζ/λ (Thr^{410/403}) (1:1000), aPKC- ζ (1:1000), or ACC (1:1000) at 4 $^\circ\text{C}$ with constant shaking. After further washing, the membranes were incubated with the secondary antibody HRP goat anti-rabbit (1:2000) for 1 h at room temperature, rinsed twice with TTBS, and once with TBS. The membranes were developed via ECL followed by exposure to Kodak

X-OMAT film. Where appropriate membranes were stripped with 0.5 M NaOH for 15 min followed by a wash with TTBS for 5 min, re-probed with GAPDH, and developed via ECL. The films were scanned using an HP Scanjet (6200C) and quantitated using Scion Image (Scion, Frederick, MD, USA). In all cases, multiple gels were analyzed and compared with control cells that had not been treated with any agents or insulin.

Calculations and statistical analysis

The rate of palmitate, oleate, and glucose uptake was calculated as the amount of radioactivity taken up by the cells in one well divided by the protein concentration (except where noted). The rate of palmitate oxidation was calculated as the amount of radioactivity recovered as $^{14}\text{CO}_2$ by the cells in one well divided by the protein concentration of the same well and corrected for label fixation using our determined acetate correction factor (6.22). All presented data are expressed as mean $\pm$ S.E.M. and as % control where control refers to cells that were not treated with insulin or inhibitors. Where appropriate, palmitate uptake and oxidation are expressed as % control where % control was calculated as the difference between the experimental and control (usually basal conditions) rates (nmol/g per min) divided by the control rate (see figure legends for specific details). The effects of treatment with insulin and/or inhibitors were analyzed using Student's *t*-test or one-way ANOVA (StatSoft Statistica 6.0; Tulsa, OK, USA) followed by Fisher LSD *post hoc* test when appropriate. In all instances, $\alpha = 0.05$ was used to determine significance.

Results

Basal palmitate and oleate uptake and oxidation

As shown in Fig. 1, both LCFA transporter proteins, FAT/CD36 and FABP_{PM}, were present in cell homogenates. Basal palmitate uptake was found to increase with palmitate concentration from 50 to 750 μM (24.8 ± 3.2 to 249.1 ± 12.0 nmol/mg for 30-min incubation) and with time of incubation, from 1 to 60 min (2.4 ± 0.1 to 55.7 ± 3.6 nmol/mg for 100 μM palmitate). Based on the linear dependency of palmitate uptake between 30 and 60 min and between 100 and 750 μM , we chose to carry out all other experiments for 30 min at a concentration of 100 μM .

Figure 1 Western blot verifying the existence of FABP_{PM} and FAT/CD36 in L6 cells. Lanes 1–6 represent L6 cells at 0, 2, 4, 10, 17, and 34 days post-confluence, and lanes 7 and 8 represent L6 cells at 10 days post-confluence.

Comparison of uptake and oxidation data between palmitate and oleate showed that both LCFA can be used as representative LCFA under our experimental conditions. LCFA uptake and oxidation rates calculated with ^{14}C -palmitate and ^{14}C -oleate are within the range of values observed by others (Palanivel & Sweeney 2005, Dimopoulos *et al.* 2006, Watt *et al.* 2006). Most importantly, our data showed that the percent increase in LCFA uptake (25 ± 0.8 vs $29.4 \pm 1.3\%$ in palmitate- and oleate-treated cells respectively) and percent decrease in LCFA oxidation (26 ± 0.3 vs $28 \pm 0.8\%$ respectively) observed with insulin stimulation were not significantly different between the two LCFA groups. This showed that the insulin effect was similar between LCFA. We chose to use ^{14}C -palmitate because its basal rates of uptake and oxidation were higher and this makes it easier to measure experimental changes.

Glucose and palmitate uptake and oxidation

To provide evidence that our cell system responds in a manner similar to L6 cell systems used by other research teams, we measured glucose uptake both in the control state and after insulin stimulation. As shown repeatedly by others (Bilan *et al.* 1991, Tsakiridis *et al.* 1995), insulin significantly ($P < 0.05$) increased glucose uptake by 30% when compared with the rate measured under control condition (554.1 ± 14.0 vs 429.9 ± 4.2 nmol/g per min). Furthermore, pretreatment with W completely abolished insulin-stimulated glucose uptake so that glucose uptake was not different from the rate measured under control condition (429.9 ± 4.2 vs 371.3 ± 6.4 nmol/g per min, $P > 0.05$).

Time and dose-response curves for insulin-stimulated palmitate uptake

Insulin, independent of glucose, significantly ($P < 0.05$) increased palmitate uptake in L6 myotubes and the insulin-induced increase (25%) was maximum at an insulin concentration of 1.0 nM (or 1×10^{-9} M which is represented as $-\log[1 \times 10^{-9}]$ M or 9 in Fig. 2A). Furthermore, to determine whether the effects of insulin were time dependent, cells were exposed to 100 nM insulin for increasing time periods, ranging from 0 to 60 min. Insulin-stimulated palmitate uptake showed a biphasic response, reaching maximal values ($P < 0.05$) at 5 and 15 min of incubation and lower values after prolonged exposure to insulin (30 and 60 min) (Fig. 2B). Therefore, to ensure maximal stimulation by insulin and for comparison purposes with other studies that typically use 100 nM insulin to stimulate the cells (Bilan *et al.* 1991, Lin *et al.* 2001), we opted to treat the cells with 100 nM insulin for 15 min for the remaining experiments.

Palmitate uptake and oxidation and insulin signaling pathway

To better characterize the insulin-induced signaling molecules involved in the regulation of insulin-mediated

Figure 2 (A) Dose-response and (B) time-response curves for insulin on palmitate uptake in L6 myotubes. Cells were exposed to (A) insulin (0–1000 nM) for 15 min or (B) 100 nM insulin for various durations (0–60 min). Palmitate uptake was measured as described in. Data are presented as means $\pm$ S.E.M. ($n=9$ per condition) and expressed as '% control' where control refers to cells not treated with insulin. * $P<0.05$ versus control or zero for both (A) and (B); & $P<0.05$ vs 0, 0.1 and 1 nM; % $P<0.05$ vs 5 and 15 min.

palmitate uptake and oxidation, PI3K, PKB/Akt, and aPKC- ζ were inhibited. Whereas PI3K inhibition with W prevented the insulin-induced increase in palmitate uptake at all three doses (Fig. 3A), PKB/Akt inhibition with A had no effect on insulin-mediated palmitate uptake at any of the doses used (Fig. 3B). aPKC- ζ inhibition with mP had no effect on insulin-mediated palmitate uptake at the lowest dose used (Fig. 3C). However, at higher doses (50 and 100 μ M), mP completely inhibited insulin-mediated palmitate uptake such that palmitate uptake was not different from their respective control conditions. To ensure that insulin stimulation activated PKB-Akt and aPKC- ζ , the phosphorylation state of these signaling molecules was measured by western blotting. PKB/Akt and aPKC- ζ protein content was not affected by treatment with insulin and/or any of the inhibitors (Fig. 4A). As shown by others (Bandyopadhyay *et al.* 1997, Hajdуч *et al.* 1998), the phosphorylation state of PKB/Akt was increased with insulin, inhibited by both W and A, and was not affected by pre-incubation with the aPKC- ζ inhibitor (mP) (Fig. 4B), suggesting that the mP compound did not interfere with insulin stimulation of PKB/Akt. Similarly, the

phosphorylation state of aPKC- ζ was increased by insulin, inhibited by both W and mP, and was not affected by pre-incubation with A (Fig. 4C). Furthermore, insulin increased PDK-1 activity as evidenced by an increase in its phosphorylation state and treatment with W prevented insulin-stimulated phosphorylation (Fig. 4D). Neither A nor mP affected the insulin-induced phosphorylation state of PDK-1. Based on the data from the dose-response curves and western blot analysis, we chose to pre-incubate the cells with 50 nM W, 10 μ M A, and 100 μ M mP for all other experiments as these concentrations maximally and specifically inhibit our target signaling molecules.

Using those selected inhibitor concentrations, our results show that inhibition of PI3K and aPKC- ζ completely abolished the insulin-induced increase in palmitate uptake such that palmitate uptake was not different from the basal rate (Fig. 5A). Conversely, PKB/Akt inhibition had no effect on the insulin-induced increase in palmitate uptake ($P>0.05$ versus insulin only condition). Furthermore, insulin significantly decreased palmitate oxidation by 29% (31.8 ± 2.4 to 24.7 ± 1.6 nmol/g per min) ($P<0.05$) (Fig. 5B). Inhibition of PI3K abolished the insulin-induced decrease in palmitate oxidation such that palmitate oxidation was not different from control ($P>0.05$), while PKB/Akt inhibition did not prevent the insulin-induced decrease in palmitate oxidation (Fig. 5B). In contrast to insulin-induced palmitate uptake, aPKC- ζ inhibition did not prevent the insulin-induced decrease in palmitate oxidation ($P<0.05$ versus control). Because the phosphorylation state of PKB/Akt under insulin-stimulated conditions was not affected by pre-incubation with mP (Fig. 4B), our results show that aPKC- ζ inhibition with mP did not prevent insulin from stimulating PKB/Akt activity. To provide a possible mechanism of action for the insulin-induced decrease in palmitate oxidation, we measured the phosphorylation state of ACC. Our data showed that ACC phosphorylation (C, 100 ± 5.2 vs I, 80 ± 15 arbitrary units) or total ACC protein was not affected by insulin (Fig. 6A and B).

Discussion

Our experiments provide extensive information about the effects of insulin, independent of its effects on glucose uptake and metabolism, on LCFA metabolism in muscle cells and, more importantly, provide novel information about the signaling molecules involved in the regulation of LCFA uptake and oxidation by insulin. Our data show that insulin, independent of glucose, directly stimulates LCFA uptake and inhibits LCFA oxidation via the PI3K-dependent insulin signaling pathway. Furthermore, our results show, for the first time, that aPKC- ζ , a signaling molecule located downstream of PI3K, is involved in the regulation of insulin-induced LCFA uptake. This notion is supported by our results that show parallel changes in aPKC- ζ activation and LCFA uptake with insulin stimulation and aPKC- ζ inhibition. Interestingly, while our results show that the insulin-induced inhibition of

LCFA oxidation is also mediated via a PI3K-dependent signaling pathway; our data suggest that PKB/Akt and aPKC- ζ are not the primary signaling intermediates involved in this insulin-induced regulation of LCFA oxidation.

Our results show that in L6 muscle cells incubated without glucose insulin stimulation increases LCFA uptake in a time- and

concentration-dependent manner. While several authors have shown previously that insulin increases LCFA uptake in incubated or perfused muscle (Muio *et al.* 1999, Dyck *et al.* 2001, Luiken *et al.* 2002), it was not clear from those results whether insulin *per se* was responsible for the observed changes in LCFA metabolism or whether the accompanying stimulatory effect of insulin on glucose uptake and metabolism was in part responsible for the measured changes. It is also interesting to note that prolonged incubation with insulin resulted in a decrease in insulin-induced LCFA uptake. These results were not unexpected since exposure to insulin for similar durations (1–8 h) has been shown to decrease glucose uptake by 20–40% in perfused rat hindquarter and incubated muscle cells (Beguino *et al.* 1986, Richter *et al.* 1988, Koivisto *et al.* 1991). As discussed by Richter *et al.* (1988), the decrease in insulin action may reflect the development of an insulin-resistant state.

The secondary aim of this study was to gather preliminary information on signaling molecules that may be involved in the regulation of LCFA uptake and oxidation by insulin. Because several of the metabolic actions of insulin on cellular metabolism have been shown to occur via stimulation of insulin receptor substrate-1-associated PI3K activity (Fares *et al.* 2001) and because data collected in incubated and perfused muscle showed that PI3K might be involved as a downstream signaling molecule in the insulin-induced pathway that regulates LCFA metabolism (Muio *et al.* 1999, Dyck *et al.* 2001, Luiken *et al.* 2002), we first determined whether PI3K activation was similarly involved in the regulation of LCFA metabolism by insulin in L6 muscle cells. In agreement with these previous results, our data show that pre-incubation of L6 muscle cells with the PI3K inhibitor wortmannin prevents the insulin-induced increase in LCFA uptake and decrease in LCFA oxidation, and that insulin-induced LCFA uptake is completely inhibited at concentrations as low as 10 nM of wortmannin. This is similar to results of Yeh *et al.* (1995) and Somwar *et al.* (1998) who found that inhibition of hexose uptake in muscle via wortmannin was concentration dependent with an IC_{50} occurring around 10–30 nM.

PI3K has been shown to activate both PKB/Akt and aPKC- ζ , two signaling molecules, which are located

Figure 3 Inhibitor dose-response curves with representative western blot for (A) wortmannin, (B) inhibitor A, and (C) mP on palmitate uptake in L6 myotubes. For inhibitor efficacy, cells were exposed to varying inhibitor concentrations for 30 min followed by insulin (100 nM) or KRB for 15 min. Palmitate uptake was measured for 30 min as described in Methods. Gel panels above figure B, C show the effects of increasing concentrations of (B) inhibitor A and (C) mP on Akt and aPKC- ζ phosphorylation. Data are presented as means $\pm$ S.E.M. ($n=6$ per condition per curve) and as '% control' where for the 'no insulin' conditions, control cells were not treated with inhibitor or insulin and were paired to the cells treated with inhibitor only and 'insulin' cells; control cells are those that have been treated with inhibitor but not insulin and were paired with cells treated with inhibitors and insulin. * $P<0.05$ versus no insulin control. † $P<0.05$ versus insulin plus 0 and 10 μM mP. A, Akt/PKB inhibitor; mP, aPKC inhibitor.

downstream of PI3K and which have been shown to be dependent upon PDK-1 for activation (Valverde *et al.* 2000, Farese 2001, Beeson *et al.* 2005, Farese *et al.* 2005). Our PDK-1 results are in agreement with the suggestion that PDK-1 lies downstream of PI3K and could possibly be a branching point for distal signaling pathways, such as Akt/PKB and aPKC- ζ (Salteil & Kahn 2001, Scheid & Woodgett 2003, Sajan *et al.* 2006, Farese *et al.* 2005). PKB/Akt has been proposed to mediate some of the cellular effects of insulin since overexpression of this signaling molecule in L6 muscle cells was shown to mimic the stimulatory actions of insulin on glucose uptake, GLUT-4 translocation to the PM, and glycogen synthesis (Hajduch *et al.* 1998, Ueki *et al.* 1998). However, our data show that PKB/Akt inhibition in L6 cells did not prevent the insulin-induced increase in LCFA uptake or decrease in LCFA oxidation and increasing the concentrations of the Akt inhibitor in the incubation medium did not change the rates of insulin-induced LCFA uptake and oxidation. Our results agree with those of Bouzakri *et al.* (2006) who showed that Akt1 and Akt2 silencing did not prevent the stimulatory effects of insulin on LCFA uptake nor the inhibitory effects of insulin on LCFA oxidation. As suggested by Bouzakri *et al.* (2006), our data show that PKB/Akt may be dispensable for the regulation of insulin-mediated LCFA uptake and oxidation in muscle cells.

A novel finding of our study is the involvement of aPKC- ζ in the regulation of insulin-mediated LCFA uptake. Direct inhibition of aPKC- ζ with an isoform-specific inhibitor commonly used in metabolic studies (Bandyopadhyay *et al.* 1997, Chabowski *et al.* 2004) completely abolished the insulin-induced increase in LCFA uptake and this effect was concentration dependent. As reported by others in cardiac myocytes (Chabowski *et al.* 2004), pre-incubation with a low concentration (10 μ M) of the aPKC- ζ inhibitor did not affect insulin-induced LCFA uptake. However, when the concentration of the inhibitor was increased to 50 or 100 μ M, the rate of insulin-induced LCFA uptake was returned to control level demonstrating the effectiveness of the inhibitor in reducing insulin signaling. This is in agreement with findings that report an IC_{50} of 48 μ M for this aPKC- ζ inhibitor with respect to insulin-stimulated glucose uptake (Bandyopadhyay *et al.* 1997). While aPKC- ζ inhibition prevented LCFA uptake, there was no effect on LCFA oxidation, suggesting that other signaling

Figure 4 Effect of insulin and PI3K, PKB/Akt, or aPKC- ζ inhibition on phosphorylation of downstream signaling molecules in L6 myotubes. Cells were exposed to inhibitors W (50 nM), A (10 μ M), and mP (100 μ M) or KRB for 30 min followed by insulin (100 nM) or KRB for 15 min and palmitate (100 μ M) for 30 min. The cells were lysed and prepared for western blotting with (A) anti-Akt or anti-aPKC- ζ (and striped and re-probed with GAPDH for control purposes) to assess total protein content that was not affected by any of the experimental conditions (see Results section), (B) phosphorylated Akt (Ser⁴⁷³), (C) phosphorylated aPKC- ζ/λ (Thr^{410/403}), or (D) phosphorylated PDK-1 (Ser²⁴¹). Data are presented as means $\pm$ S.E.M. ($n=6$ per condition). * $P<0.05$ versus control and insulin plus inhibitor conditions. W, PI3K inhibitor, wortmannin; A, Akt/PKB inhibitor; mP, aPKC inhibitor.

Figure 5 Effect of insulin and PI3K, Akt/PKB, or aPKC ζ inhibition on (A) palmitate uptake and (B) oxidation in L6 myotubes. Cells were exposed to inhibitors W (50 nM), A (10 μ M), and mP (100 μ M) or KRB for 30 min followed by insulin (100 nM) or KRB for 15 min and [14 C]palmitate (100 μ M) for 30 min. Palmitate uptake and oxidation were measured as described in Methods. Data are presented as means $\pm$ S.E.M. ($n=12-18$ per condition, $n=6$ per condition per curve); and as '% control' where control refers to paired cells not treated with insulin. * $P<0.05$ versus control (no insulin, no inhibitors); # $P<0.05$ versus insulin only. W, PI3K inhibitor, wortmannin; A, Akt/PKB inhibitor; mP, aPKC inhibitor.

mechanisms may be mediating these effects. It is known that the MAPK/ERK1/2 pathway is involved in insulin signaling and may mediate some of the effects of insulin on intracellular lipid metabolism. Indeed, our laboratory has recently demonstrated that ERK1/2 is involved in the regulation of contraction-induced changes in LCFA metabolism (Raney & Turcotte 2007). Whether ERK1/2 activation plays a similar regulatory role during insulin stimulation in L6 cells is not known. Finally, it might also be argued that the insulin-mediated decrease in LCFA oxidation is regulated metabolically via changes in enzyme activity. It has been shown by some (Witters & Kemp 1992, Gamble & Lopaschuk 1997) but not others (Winder & Holmes 2000) that insulin activates ACC. In line with a well-documented chain of metabolic events, ACC stimulation would be associated with high intracellular malonyl-CoA and inhibition of carnitine palmitoyltransferase-1 and ultimately lead to a decrease in LCFA oxidation. However, our ACC data show that under our experimental conditions, metabolic regulation of insulin-mediated LCFA oxidation via this scenario was not significant.

Figure 6 Effect of insulin on ACC (Ser⁷⁹) phosphorylation in L6 myotubes. Cells were exposed to insulin (100 nM) for 15 min prior to exposure to unlabelled palmitate (100 μ M) for 30 min. Cells were lysed and prepped for western Blot with (A) phosphorylated ACC (Ser⁷⁹) and (B) total ACC. Total ACC membranes were stripped and re-probed with GAPDH as described in Methods. Data are presented as arbitrary units $\pm$ S.E.M. ($n=4$ gels per condition).

In conclusion, our results demonstrate for the first time that insulin, independent of glucose, increases LCFA uptake and decreases LCFA oxidation via insulin-induced PI3K signaling in muscle cells. Furthermore, our data show that insulin exerts its stimulatory effect on LCFA uptake via the PI3K-aPKC- ζ branch of the insulin-PI3K signaling cascade. While our data are based on the information obtained using chemical inhibitors and cannot be used exclusively and absolutely as proof of our hypothesis, our results suggesting the involvement of aPKC- ζ in the regulation of LCFA uptake by insulin points the direction for future, more sophisticated, studies utilizing constitutively active and inactive kinases and/or siRNA.

Declaration of Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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