Research Communication

Acute and long-term administration of palmitoylcarnitine induces muscle-specific insulin resistance in mice

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Abstract

Acylcarnitine accumulation has been linked to perturbations in energy metabolism pathways. In this study, we demonstrate that long-chain (LC) acylcarnitines are active metabolites involved in the regulation of glucose metabolism *in vivo*. Single-dose administration of palmitoylcarnitine (PC) in fed mice induced marked insulin insensitivity, decreased glucose uptake in muscles, and elevated blood glucose levels. Increase in the content of LC acylcarnitine induced insulin resistance by impairing Akt phosphorylation at Ser473. The long-term administration of PC using slow-release osmotic minipumps induced marked hyperinsulinemia, insulin resistance, and glucose intolerance, suggesting that the permanent accumulation of LC acylcarnitines can accelerate the progression of insulin resistance. The decrease of acylcarnitine content significantly improved glucose tolerance in a mouse model of diet-induced glucose intolerance. In conclusion, we show that the physiological increase in content of acylcarnitines ensures the transition from a fed to fasted state in order to limit glucose metabolism in the fasted state. In the fed state, the inability of insulin to inhibit LC acylcarnitine production induces disturbances in glucose uptake and metabolism. The reduction of acylcarnitine content could be an effective strategy to improve insulin sensitivity.

Keywords: *acylcarnitine; insulin resistance; insulin release; fatty acid metabolism; glucose metabolism; isopropyl-GBB*

Abbreviations: *ACC, acetyl-CoA carboxylase; ACSL, long-chain fatty acid CoA synthetase; AMPK, AMP-activated protein kinase; CPT-1, carnitine palmitoyltransferase-1; [^{3}H]-DOG, 2-[1,2-^{3}H]-deoxy-d-glucose; FA, fatty acid; GB, guanabenz; GLUT1 and 4, glucose transporter 1 and 4; HFD, high-fat diet; IL1β, interleukin 1β; iNOS, inducible nitric oxide synthase; IP-GBB, 4-[isopropyl(dimethyl)ammonio]butanoate; LC, long chain; MCD, malonyl-CoA decarboxylase; OCTN2, organic cation transporter 2; PC, palmitoylcarnitine; PDH, pyruvate dehydrogenase; PDK4, pyruvate dehydrogenase lipoamide kinase isozyme 4; TNFα, tumor necrosis factor α.*

Additional Supporting Information may be found in the online version of this article.

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1. Introduction

Insulin resistance, as the main feature of type 2 diabetes, involves inappropriate glucose metabolism. It has been suggested that insulin resistance and disturbances in glucose metabolism is induced by excessive fatty acid (FA) flux which results in incomplete FA oxidation and the accumulation of various lipid metabolites, including free FA, diacylglycerols, ceramides, acyl-CoAs, and acylcarnitines. Among FA intermediates linked to insulin resistance are long-chain (LC) acylcarnitines [1,2]. The CPT-1-catalyzed synthesis of LC acylcarnitines is an important step in LC FA uptake in mitochondria [3]. Generally, CPT-1 has been known only as a step in the mitochondrial FA oxidation, and the accumulation of LC acylcarnitines has been seen as marker of incomplete mitochondrial metabolism of FA. However, taking into account the LC acylcarnitine-induced effects on Akt phosphorylation [4,5], an increase in LC acylcarnitine content could be considered as a feedback inhibition mechanism of insulin action. In this way, insulin- and AMPK-mediated regulation of CPT-1 activity would have physiological meaning, and LC acylcarnitines would emerge as active metabolites important for the regulation of energy metabolism.

It has been determined that plasma and skeletal muscle concentrations of LC acylcarnitines are modestly increased among individuals with insulin resistance and type 2 diabetes mellitus [6,7]. Recently, it was shown that during insulin stimulation, plasma levels of LC acylcarnitines reflect age-related metabolic dysfunction [8]. LC acylcarnitines are very active and effectively inhibit pyruvate and lactate oxidation in mitochondria, thus, compromising glucose uptake and oxidation in models of isolated mitochondria [9], cell culture [5], and isolated heart tissue [9,10]. It was hypothesized that LC acylcarnitines ensure the inhibition of glucose metabolism in order to avoid hypoglycemia and gain energy from unlimited lipid stores [9]. Thus, considering LC acylcarnitines as important players in metabolism, it is worthwhile to study their role in the development of insulin resistance.

In this study, we used both LC acylcarnitine administration and pharmacologically mediated reduction in acylcarnitine content to test whether the LC acylcarnitine content in mice muscle could influence glucose metabolism and induce the development of insulin resistance. To increase the LC acylcarnitine content, both single-dose and long-term administration of palmitoylcarnitine (PC), as the most prominent LC acylcarnitine, were used. We used osmotic minipumps to continuously deliver PC. A novel compound, 4-[isopropyl(dimethyl)ammonio]butanoate (IP-GBB), which is a potent inhibitor of L-carnitine transport by organic cation transporter 2 (OCTN2) [11,12], was used to decrease the acylcarnitine content in a model of diet-induced glucose intolerance. Our study is intended to explain the physiological role of LC acylcarnitines and to link the acceleration of skeletal muscle insulin resistance to the excessive accumulation of LC acylcarnitines.

2. Experimental Procedures

2.1. Animals and treatments

A total of 180 male CD-1 (12 weeks old, Harlan Laboratories BV and Envigo) mice were housed under standard conditions (21–23°C, 12 h light–dark cycle) with unlimited access to food (R70 diet, Lantmännen Lantbruk, Sweden) and water. All animals were adapted to local conditions for 2 weeks before the start of experiments. The experimental procedures were carried out in accordance with the guidelines of the European Community, local laws and policies and were approved by the Latvian Animal Protection Ethical Committee, Food and Veterinary Service, Riga, Latvia. All studies involving animals are reported in accordance with the ARRIVE guidelines [13,14]. Adult CD-1 mice were used for experiments as a well characterized strain widely used for metabolic studies. In addition, adult male Wistar rats ($n = 10$) were used to characterize guanabenz induced hypoinsulinemia/hyperglycemia state at multiple time points. Animals were randomized according to their body weight in order to match the age of control and experimental groups. All samples were collected in the morning 9–11 A.M.

2.2. Single-dose PC administration

PC hydrochloride was synthesized from L-carnitine and palmitoyl chloride by a modified protocol (see Supporting Information) as described in the literature [15]. To study the effects of LC acylcarnitines on glucose homeostasis, PC was administered intraperitoneally at a dose of 50 mg/kg. In the fasted state, glucose and insulin levels were measured 60 min after PC administration. In the fed state, after 30 min of PC administration, insulin (0.3 IU/kg) was administered subcutaneously, and glucose concentrations were measured 60 min after PC administration. In addition, to ensure a continuous dosing of PC, osmotic minipumps (ALZET®, USA) filled with PC (50 mg/kg/day) were implanted subcutaneously in the mice ($n = 4$) for 24 h. In control animals ($n = 4$), osmotic minipumps loaded with saline (vehicle) were implanted. On the day prior to ALZET minipump implantation, mice were anesthetized using 2% isoflurane dissolved in the mixture of oxygen and nitrous oxide (50/50, v/v). Fur from the scapular region and nape was removed using commercially available depilation cream. On the next day, experimental animals received sodium benzylpenicillin (150 mg/kg i.p.) and tramadol (10 mg/kg i.p.) and were anesthetized using 2% isoflurane. Transverse 1.5 cm skin incision was made above shoulder blades and subcutaneous pocket was formed on the back of the animal. ALZET minipump model 2004 was inserted in the subcutaneous pocket and skin was closed using 4–0 silk sutures (Ethicon, USA). Glucose tolerance testing and metabolic phenotyping was performed during 24 h after the implantation of minipumps. To inhibit endogenous insulin release, guanabenz (i.p. 1 mg/kg, GB), an α_2-adrenoreceptor agonist [16], was used after the single-dose PC (50 mg/kg) administration. In fasted animals, glucose flux is very limited and GB induces hyperglycemia only

in fed animals, therefore in our experiment, we administrated GB in fed mice. Thirty mice were randomly separated into two experimental groups, GB ($n = 15$) and GB + PC ($n = 15$). To determine the insulin-dependent effects of PC, insulin (0.3 IU/kg) was administered subcutaneously 1 h after the intraperitoneal injection of GB or GB + PC.

2.3. Slow-release and long-term PC administration

To determine the effects of the long-term increased availability of LC acylcarnitines, mice were randomly separated into control ($n = 10$) and PC ($n = 10$) groups. To ensure a continuous and long-term dosing of PC, osmotic minipumps (ALZET®, USA) filled with PC (10 mg/kg/day) were implanted subcutaneously in the mice for 28 days. In control animals, osmotic minipumps loaded with saline (vehicle) were implanted. The osmotic minipump implantation was performed identically as described above in the single-dose PC administration section. Sutures were removed on the seventh day after the surgery. At the end of the 28-day treatment, glucose and insulin tolerance tests were performed, and biochemical parameters were measured.

2.4. Pharmacological decrease of LC acylcarnitines

To determine the therapeutic potential of a decreased availability of LC acylcarnitines on insulin resistance, the pharmacological agent IP-GBB at a dose of 10 mg/kg which decreases acylcarnitine content, was used in the study. Synthesis of IP-GBB was performed as described by Tars et al. [12]. Thirty mice were randomly separated into three experimental groups, R70 Control ($n = 10$), HFD (Western RD, Special Diets Services, UK) ($n = 10$), and HFD + IP-GBB ($n = 10$). At the end of the 28-day treatment, glucose (1 g/kg) and insulin (0.5 IU/kg) tolerance tests were performed, and biochemical parameters were measured.

2.5. Cell culture experiments

To assess the effect of PC on insulin secretion *in vitro*, we used an insulin-secreting cell line, RIN-5F (ATCC® Number: CRL-2058) and determined the insulin concentration in the culture medium using a Sensitive Rat Insulin RIA kit (Millipore, Billerica) after 1 h of incubation with PC at a dose of either 3 or 10 μM in serum and FA-free DMEM under both basal and hyperglycemic conditions, which contained 5.5 and 11 mM glucose, respectively. To differentiate the PC and palmitic acid-induced effects on insulin secretion, DC260126, a small-molecule antagonist of free fatty acid receptor 1 (FFAR1/GPR40), at a concentration of 5 μM was preincubated 1 h before the addition of PC at a concentration of 10 μM.

To evaluate the PC-induced effect on Akt phosphorylation, C2C12 ATCC® CRL-1772™) mouse skeletal myoblasts were differentiated in DMEM containing 2% horse serum for 5 days. Then, the medium was replaced with serum-free DMEM and PC at doses of 5 and 10 μM; after an overnight incubation, the basal or insulin-stimulated (10 nM for 15 min) level of Akt phosphorylation was determined by a western blot analysis. The medium was removed, and the cells were lysed in a buffer containing 100 mM Tris–HCl, pH 7.4, 10 mM EDTA, 5 mM $MgCl_2$, 1 mM glycerol 3-phosphate, 1 mM NaF, 500 μM Na_3VO_4, 1 mM DTT, phosphatase inhibitor cocktail I 1:100 (Alfa Aesar), protease inhibitors (10 μM leupeptin, 1 μM pepstatin, 1 μM aprotinin, and 100 μM PMSF), and 1% IGEPAL at 4°C for 10 min. The lysates were centrifuged at 10,000 rpm for 5 min at 4°C. The supernatants were stored at −80°C until analysis.

2.6. Measurements of glucose uptake and fatty acid metabolism *in vivo*

To determine the glucose uptake or FA metabolism (uptake and oxidation) *in vivo*, 1 μCi of 2-[1,2-^{3}H]-deoxy-D-glucose ([^{3}H]-DOG, specific activity, 60 Ci/mmol) or 1 μCi [9,10-^{3}H]-palmitate (specific activity, 60 Ci/mmol), respectively, were administered intravenously to the mice. After 10 min, the mice were sacrificed by cervical dislocation, and skeletal muscle, liver and adipose tissue homogenates (1:5, w/v in MilliQ water) were prepared. To determine the palmitate metabolism *in vivo*, samples were treated as previously described [17]. The contents of [^{3}H]-DOG or [^{3}H]-palmitate in the tissue samples were determined by a liquid scintillation method.

2.7. Metabolic phenotyping

The PhenoMaster system for mice (TSE, Germany), with automated food/liquid access control units and an indirect gas calorimetry system, was used to monitor 24 h changes in energy metabolism. Indirect gas calorimetry was used for the measurements of the animals' oxygen consumption (VO_2) and carbon dioxide production (VCO_2) to estimate various metabolic parameters, including the respiratory exchange rate (RER) and fat and carbohydrate utilization for energy production.

2.8. mRNA isolation and quantitative RT-PCR analysis

Total RNA from muscle tissues was isolated using TRI Reagent (Sigma, St. Louis, MO) according to the manufacturer's protocol. The first-strand cDNA synthesis was carried out using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Foster City, CA) following the manufacturer's instructions. The quantitative RT-PCR analysis of gene expression was performed by mixing SYBR® Green Master Mix (Applied Biosystems™), synthesized cDNA, forward and reverse primers specific for glucose transporter 1 (GLUT1) and 4 (GLUT4), carnitine palmitoyltransferase-1A (CPT-1A) and −1B (CPT-1B), long-chain fatty acid CoA synthetase (ACSL), pyruvate dehydrogenase lipoamide kinase isozyme 4 (PDK4), tumor necrosis factor α (TNFα), interleukin 1 β (IL1β), inducible nitric oxide synthase (iNOS), or β-actin and running the reactions on an Applied Biosystems Prism 7500 instrument according to the manufacturer's protocol. The relative expression levels for each gene were calculated with the $\Delta\Delta C_t$ method and normalized to the expression of β-actin. The primer sequences used for the quantitative RT-PCR analysis are available upon request.

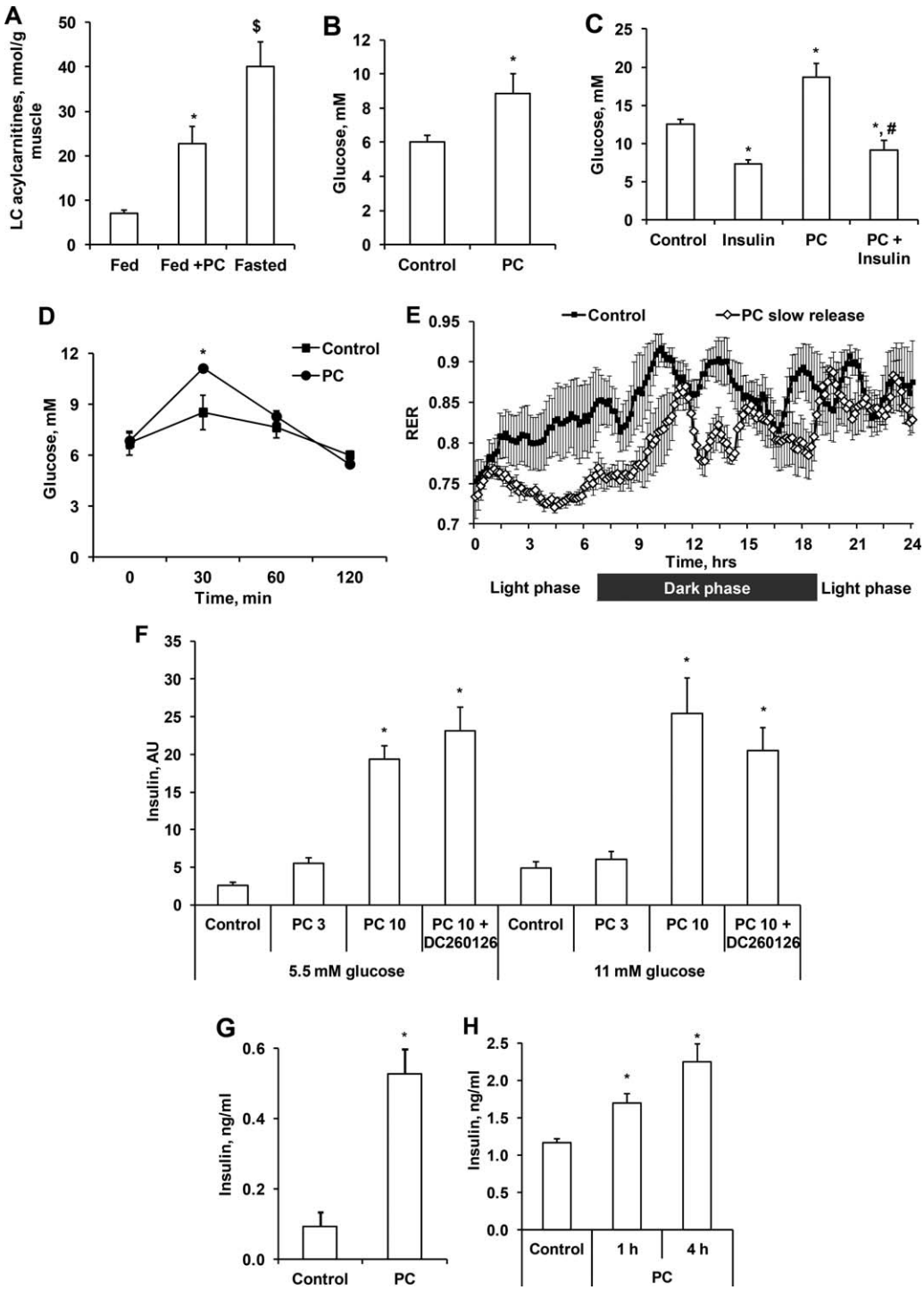


FIG 1

Acute PC effects on the concentrations of blood glucose and insulin and ^{3}H-deoxyglucose uptake. The administration of PC (50 mg/kg i.p. 1 h) increased the muscle long-chain acylcarnitine content (A), the blood glucose concentrations in fasted (B), and fed (C) mice in vivo*, while the administration of insulin (0.3 IU/kg, s.c.) overcame the PC-induced effects on blood glucose (C). The administration of PC (50 mg/kg/24 h) with osmotic minipumps induced disturbances in glucose tolerance (D) and a delayed shift of energy production toward glucose oxidation (E). PC (3 and 10 μM 1 h) induced insulin release in insulin-secreting cell line, RIN-5F, which was not inhibited by an inhibitor of FA targeted GPR40 (FFAR1) receptor (F). The administration of PC 50 and 100 mg/kg after 1 h induced an increase in insulin release, respectively, in fasted (G) and fed state (H)* in vivo*. Guanabenz (GB; 1 mg/kg i.p.) administration significantly reduced plasma insulin concentration (I) and induced a marked increase in glucose concentration, which was reduced by the administration of insulin (0.3 IU/kg s.c.) (J). The administration of PC (50 mg/kg i.p.) with GB and insulin markedly diminished the insulin action on blood glucose level (J) and [^{3}H]-DOG uptake in skeletal muscles (K), while it did not influence the uptake and metabolism of [^{3}H]-palmitate (L). Palmitate metabolism was determined by measurement of acid soluble species (ACS). Each value represents the mean ± SEM of 4 (D, E), 5 or 8 (experiments with GB) animals. For A, each value represents the mean ± SEM of 8 (Fed), 3 (Fed + PC), or 5 (fasted) animals.*Significantly different from the respective control group, $^{\#}$significantly different from the insulin control group, and $^{\$}$significantly different from the baseline (Student's* t *test or ANOVA following Tukey's test,* $P < 0.05$*).*

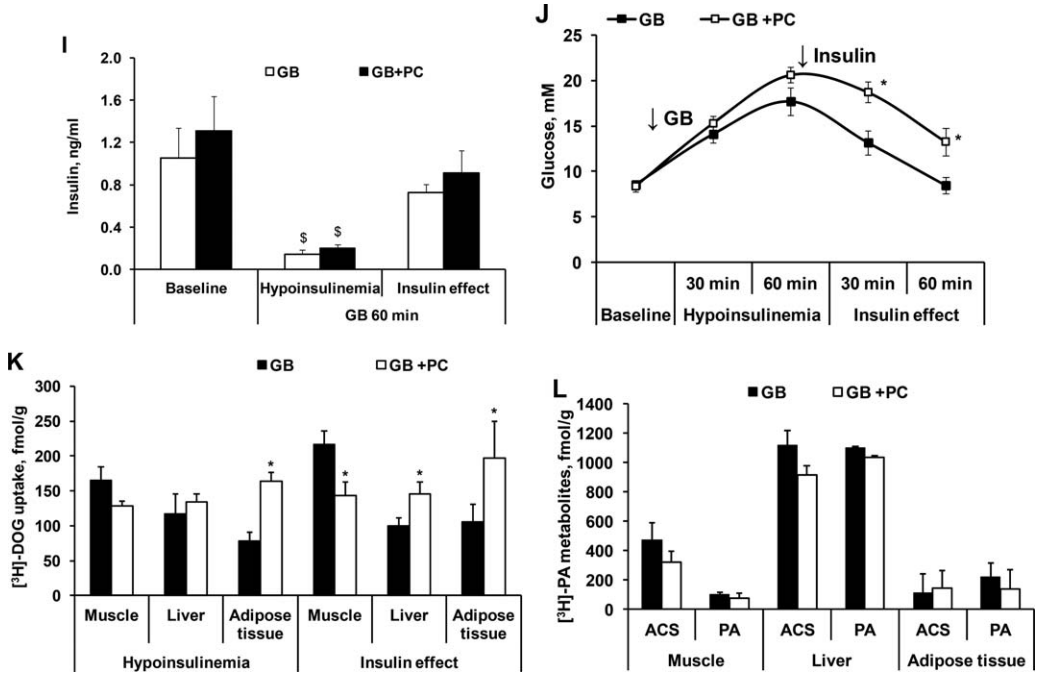


FIG 1 *(Continued)*

2.9. Western blot analysis of tissue lysates

Hindlimb muscle tissues were homogenized by an Ultra-Turrax® homogenizer (IKA, Germany) at a ratio of 1:10 (w/v) at 4°C in a buffer containing 100 mM Tris–HCl, pH 7.4, 10 mM EDTA, 5 mM $MgCl_2$, 1 mM glycerol 3-phosphate, 1 mM NaF, and protease inhibitors (10 μM leupeptin, 1 μM pepstatin, 1 μM aprotinin, and 100 μM PMSF). The PAGE and western blot analysis of tissue lysates was performed as described previously [18]. To detect the phosphorylation level of Akt at Ser473, membranes were incubated with anti-P-Akt (#sc-7985-R; Santa Cruz Biotechnology, CA, or #4060S; Cell Signaling Technology, Danvers, MA) specific antibodies and the obtained data were normalized against total Akt (#sc-8312; Santa Cruz Biotechnology) protein expression. The blots were developed using chemiluminescence reagents (Millipore). The western blot images were scanned and then analyzed using Gel-Pro Analyzer 6.0 software.

2.10. Pyruvate dehydrogenase activity assay

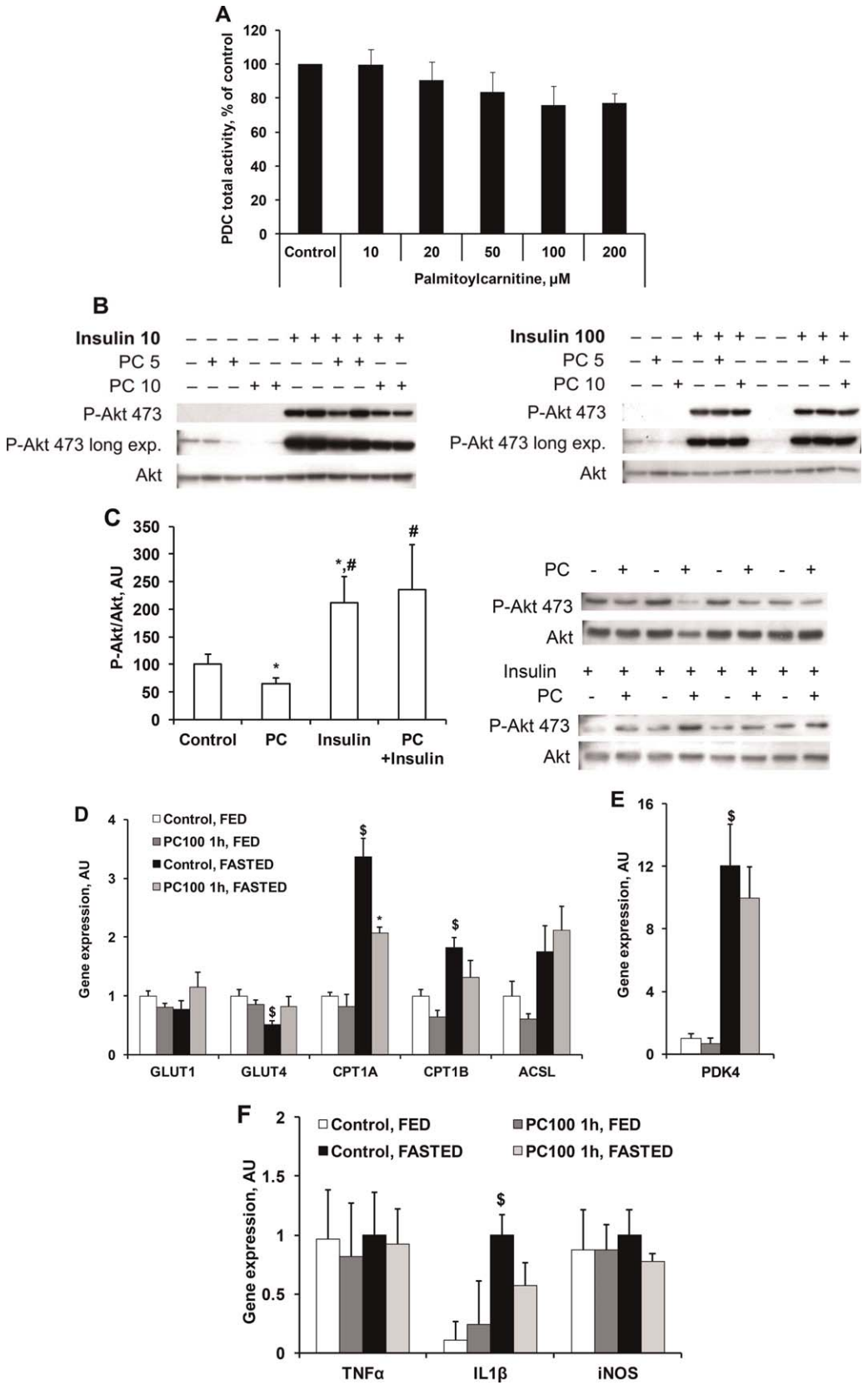
Isolated cardiac mitochondria were used to determine the ability of PC to inhibit pyruvate dehydrogenase (PDH) a rate limiting enzyme of PDH complex. PC was preincubated with the isolated mitochondria for 10 min at room temperature. The activity of PDH was measured spectrophotometrically as previously described [19].

2.11. Determination of acylcarnitine profile in muscles

Determination of the content of acylcarnitines in the skeletal muscle tissue samples was performed by the ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS-MS) as described by Kivilompolo et al. [20] with some modifications. Waters Acquity UPLC H-Class chromatograph was coupled to Waters Xevo TQ-S tandem mass spectrometer. Chromatographic separation was performed on Waters Acquity UPLC BEH Hilic (2.1 × 100 mm, 1.7 μm) column in gradient mode. Solvent A was 10 mM ammonium acetate with 0.2% formic acid in water and solvent B was acetonitrile. Initial mobile phase composition was 10% solvent A and was linearly increased to 20% solvent A in 7 min. Total run time with column flush and re-equilibration was 10 min. Column temperature was 30°C and flow rate was 0.5 mL/min. Data acquisition was performed in positive electrospray ionization (ESI+) and multiple reaction monitoring (MRM) mode. The ion source parameters were as follows: source temperature 120°C, capillary voltage 2.5 kV, desolvation gas temperature 600°C, and desolvation and cone gas flow 800 L/h and 150 L/h, respectively. The MRM transitions with cone and collision energy values are presented in Supporting Information Table S1. The sample extraction was performed as described previously [21,22]. The concentrations of acylcarnitines were measured against a nine-point standard curve of C4–C18 and C2–C3 acylcarnitine in a range 0.01–20 nM and 0.2–200 nM in analytical sample, respectively. The concentration of acylcarnitine was expressed nmol per mg of wet weight.

2.12. Glucose and insulin tolerance tests

To perform the glucose tolerance test, the mice were fasted overnight. Then, a glucose solution (0.5 or 1 g/kg of body weight) was administered intraperitoneally, and blood samples


A
PDC total activity, % of control
120
100
80
60
40
20
0
Control
10
20
50
100
200
Palmitoylcarnitine, μM
B
Insulin 10
PC 5
PC 10
P-Akt 473
P-Akt 473 long exp.
Akt
Insulin 100
PC 5
PC 10
P-Akt 473
P-Akt 473 long exp.
Akt
C
P-Akt/Akt, AU
350
300
250
200
150
100
50
0
*
*,#
#
Control
PC
Insulin
PC +Insulin
PC
P-Akt 473
Akt
Insulin
PC
P-Akt 473
Akt
D
Control, FED
PC100 1h, FED
Control, FASTED
PC100 1h, FASTED
Gene expression, AU
4
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1
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GLUT1
GLUT4
CPT1A
CPT1B
ACSL
E
Gene expression, AU
16
12
8
4
0
$
PDK4
F
Control, FED
PC100 1h, FED
Control, FASTED
PC100 1h, FASTED
Gene expression, AU
2
1.5
1
0.5
0
$
TNFα
IL1β
iNOS

were drawn from the tail vein at 0 (fasting), 15, 30, 60, 120, 180, and 240 min. To perform the insulin tolerance test, an insulin solution (0.3 or 0.5 IU/kg of body weight) was administered subcutaneously to the fed mice, and blood samples were drawn from the tail vein at 0 (fed), 30, 60, 120, and 240 min. The blood glucose concentration was measured using a MediSense Optium Xceed blood glucose meter and strips.

2.13. Determination of biochemical parameters in plasma

For biochemical measurements, blood samples were collected from the tail vein in heparin-containing tubes. To obtain plasma, the samples were centrifuged at 1,000*g* for 10 min at 4°C. All samples were stored at −80°C until analysis. The plasma glucose, triglyceride, and insulin concentrations were determined using kits from Instrumentation Laboratory, a Sensitive Rat Insulin RIA kit and a Rat/Mouse Insulin ELISA kit (Millipore). The lactate level was measured in the samples using an enzymatic kit from Roche Diagnostics (Mannheim, Germany). The concentration of free FAs was measured using commercially available enzymatic kit from Wako (Neuss, Germany).

2.14. Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM). For the statistical analyses, Student's *t* test or a one-way ANOVA with Tukey's post-test were used. *P* values less than 0.05 were considered to be statistically significant. The statistical calculations were performed using Prism 3.0 software (GraphPad, San Diego, California).

3. Results

3.1. Effects of single-dose PC administration on glucose metabolism

Acylcarnitines are intermediates of FA metabolism, but they constitute only a small fraction of total FA pool. Administration of PC at doses of 10–50 mg/kg/24 h corresponds to only 0.3–1.5% of the total body FA turnover suggesting that administration of PC does not significantly influence FA metabolism. In addition, PC transport in the skeletal muscles is limited and administration of PC at a dose of 50 mg/kg induced only a threefold increase in the intramuscular content of LC acylcarnitines (Fig. 1A and Supporting Information Table S2). In comparison, after overnight fasting skeletal muscle LC acylcarnitine content was fivefold higher than LC acylcarnitine content in fed state (Fig. 1A, Supporting Information Table S2). Thus, PC treatment induced effect on muscle LC acylcarnitine content is similar to short-term fasting.

The single-dose administration of PC induced a significant increase in the blood glucose concentrations of fasted (Fig. 1B) and fed (Fig. 1C) mice. The administration of insulin significantly reduced blood glucose with and without the coadministration of PC. This additional increase in insulin concentration was able to decrease the PC-induced effect on blood glucose, suggesting that higher concentrations of insulin can overcome PC-induced effects on glucose metabolism.

To test the 24-h effect of PC administration on glucose tolerance and energy metabolism balance in mice, PC (50 mg/kg/day) was administered by slow-release minipumps. In the glucose tolerance test, we observed a significantly higher increase in blood glucose in the PC group mice (Fig. 1D). In addition, the RER was decreased in mice with PC (Fig. 1E). Thus, increased PC concentration delays the glucose metabolism transition from the fed to fasted state.

Taking into account the LC acylcarnitine-induced effects on insulin secretion observed in an *in vitro* model [23], we determined the effect of PC on insulin release *in vitro* and *in vivo*. According to the study by Soni et al., insulin-stimulating action depends on intracellular LC acylcarnitine production and transport rate into cells [23]. We also found that 3 and 10 μM concentrations of PC were sufficient to increase intracellular LC acylcarnitine contents within 1 h and to stimulate insulin release from RIN-5F cells (Fig. 1F). To evaluate whether PC acts by a different mechanism than FA, we incubated PC in the presence of DC260126, an inhibitor of FA targeted GPR40 (FFAR1) receptor in β-cells. The effect of 10 μM PC was not inhibited by DC260126 (Fig. 1F) indicating that observed PC effect on insulin release was not induced by palmitate action. To confirm this effect *in vivo*, we performed a bolus administration of PC in fasted mice. The administration of PC induced a significant fivefold increase in insulin concentration (Fig. 1G). Up to a twofold increase in insulin concentration was also observed after PC administration in fed mice (Fig. 1H). Overall, PC potentiates glucose-stimulated insulin release, thus suggesting that the LC acylcarnitine effect is important for the physiological transition from the fasted to fed state and that it induces hyperinsulinemia in the case of diabetes.

Serious drawback of insulin clamp method in mice is necessity to administrate general anesthetics which induce marked

FIG 2

Mechanisms of PC action. PC does not influence pyruvate dehydrogenase (PDH) activity in isolated mitochondria (A). In C2C12 cells, the basal phospho-Akt level is very low, while insulin-stimulated Akt phosphorylation is pronounced, and PC at a concentration of 5 and 10 μM dose-dependently decreased the level of phospho-Akt (B). Insulin at 100 nM overcame PC-induced effect on Akt phosphorylation (B). The administration of PC (50 mg/kg i.p. 1 h) decreased the level of phospho-Akt in fed mouse muscle in vivo*, while the administration of insulin (0.3 IU/kg s.c.) overcame the PC-induced effect (C). Significant changes were observed in gene expression between the fed and fasted states, while PC administration (100 mg/kg i.p. 1 h) did not influence the expression of selected genes of energy metabolism (D, E) and inflammation (F). Each value represents the mean ± SEM of five animals or three to five independent measurements of PDH activity. *Significantly different from the control group and $^{\#}$significantly different from the PC group (ANOVA following Tukey's test, $P < 0.05$). $^{\$}$Significantly different from the fed control group (ANOVA following Tukey's test, $P < 0.05$).*

insulin resistance [24]. Therefore, taking into account the PC effect on insulin release, we used a novel experimental method suitable for the evaluation of both insulin-dependent and insulin-independent glucose homeostasis *in vivo*. To study the insulin-independent PC effects on plasma glucose, we administered the α_2-adrenoreceptor agonist GB, which inhibits insulin release [16,25]. For the evaluation of insulin-dependent effects, GB administration was followed by insulin administration. In the fed control mice, administration of GB caused hypoinsulinemia (Fig. 1I) and induced a marked increase in plasma glucose from 8.6 mM up to 17.7 mM, while the subsequent insulin administration increased insulin concentration (Fig. 1I) and significantly decreased the glucose concentration back to the initial level (Fig. 1J). The administration of PC in combination with GB induced increase in the plasma glucose concentration to 3 mM higher than that in the GB group. PC also significantly diminished the insulin-induced blood glucose lowering effect (Fig. 1J). Thus, the blood glucose concentration in the PC group remained 5 mM higher than that in the control mice. In rats the PC-induced effect on insulin-dependent blood glucose utilization was even more pronounced and glucose concentration did not decrease after administration of insulin (Supporting Information Fig. S1). These effects could be explained by a PC-induced significant decrease in insulin-stimulated [^{3}H]-DOG uptake in skeletal muscles (Fig. 1K). Importantly, the PC effect on blood glucose is partially masked by the significantly stimulated [^{3}H]-DOG uptake in liver and adipose tissue (Fig. 1K). In addition, no effect of PC on [^{3}H]-palmitate uptake and metabolism was observed (Fig. 1L). Overall, the PC administration limits insulin-related glucose uptake in muscles.

3.2. Mechanisms of PC action

In previous studies, the inhibition of the PDH was suggested as a possible mechanism of PC action [9,26]. In this study, we did not observe any significant PC effect on PDH activity (Fig. 2A). The overnight incubation of PC (5 or 10 μM) with or without insulin (10 nM for 15 min) decreased Akt Ser-473 phosphorylation in the C2C12 muscle cell line (Fig. 2B). The increase in insulin concentration overcomes the PC-induced inhibition of Akt phosphorylation (Fig. 2B). In the C2C12 cell line, incubation with PC in the presence of 100 nM insulin did not influence the phospho-Akt Ser-473 levels (Fig. 2B). A similar effect on Akt phosphorylation was observed in mouse muscles after a single administration of PC (50 mg/kg) (Fig. 2C). Thus, PC induces the inhibition of Akt phosphorylation, while increases in insulin concentration up to a certain level can overcome the PC-induced effects on insulin signaling.

It would be expected that administered PC competes with FA for uptake and oxidation in mitochondria. However, PC does not influence the FA uptake and metabolism rate in skeletal muscle tissue (Fig. 1L). Taking into account that FA metabolism was not influenced by PC, the inhibition of glucose metabolism is also not related to the Randle cycle and excessive mitochondrial oxidation of PC. In addition, PC administration does not influence PPARα activity (data not shown) and the expression of genes involved in muscle glucose transport and FA metabolism (Figs. 2D and 2E) and genes related to inflammation (Fig. 2F). We observed significant differences in gene expression between the fed and fasted states; however, PC did not influence FA metabolism-related and inflammatory gene expression in either state. Overall, the inhibitory effect on Akt phosphorylation and related insulin signaling is an important mechanism of PC action.

3.3. Effects induced by the long-term, slow-release administration of palmitoylcarnitine

To ensure the long-term administration of PC, we used slow-release osmotic minipumps (ALZET®, USA). This experimental setup ensured a permanent increase in LC acylcarnitine content in muscles for 28 days. In the control mice, minipumps loaded with saline (vehicle) were implanted. PC administration at a dose of 10 mg/kg/day in muscles induced a substantial twofold increase in the content of PC and total LC acylcarnitine content (Figs. 3A and 3B, Supporting Information Table S3). Increase in content of other LC acylcarnitines could be explained by the inhibitory action of PC on the insulin signaling pathway, which in turn limits the Akt-mediated inhibition of CPT-1 activity. In addition, PC competed for metabolism in mitochondria with other LC acylcarnitines and induced increases in their content.

A long-term increase in LC acylcarnitine content resulted in impaired glucose tolerance (Fig. 3C). The AUC calculated from the glucose tolerance test data in the PC group was significantly increased by 30%. PC administration by minipumps completely blocked the insulin-induced blood glucose lowering effect in the insulin tolerance test (Fig. 3D). In the control animals, insulin administration induced an approximate 3 mM decrease in blood glucose concentration, while in the PC group, blood glucose was not changed during the 4 h test period. In PC-treated mice, plasma glucose concentrations were similar to that of the control mice; however, it is likely that glucose concentrations were not affected due to the 2.8-fold increase in insulin concentration in the fed state (Fig. 3E). In addition, the PC-induced increase in lactate concentration suggests incomplete glucose oxidation (Fig. 3F). In contrast, PC administration did not influence plasma triglyceride and FA concentrations (Figs. 3G and 3H). PC administration with slow-release capsule did not change mice weight gain (Fig. 3I). Thus, the PC-induced glucose intolerance is not related to changes in FA flux. Overall, the long-term increase in LC acylcarnitine content induced insulin resistance and hyperinsulinemia.

3.4. Effect induced by the administration of an OCTN2 inhibitor in HFD-fed mice

To confirm our hypothesis that a decrease in LC acylcarnitine content could improve insulin sensitivity in insulin-resistant individuals, we used a high-fat diet (HFD)-induced insulin-resistance model and a novel inhibitor of OCTN2, IP-GBB. In HFD-fed mice, the muscle content of short-, medium- and LC acylcarnitines was similar to that of control mice (Fig. 4A, Supporting Information Table S4), while a 28-day treatment with IP-GBB significantly decreased the LC acylcarnitine content

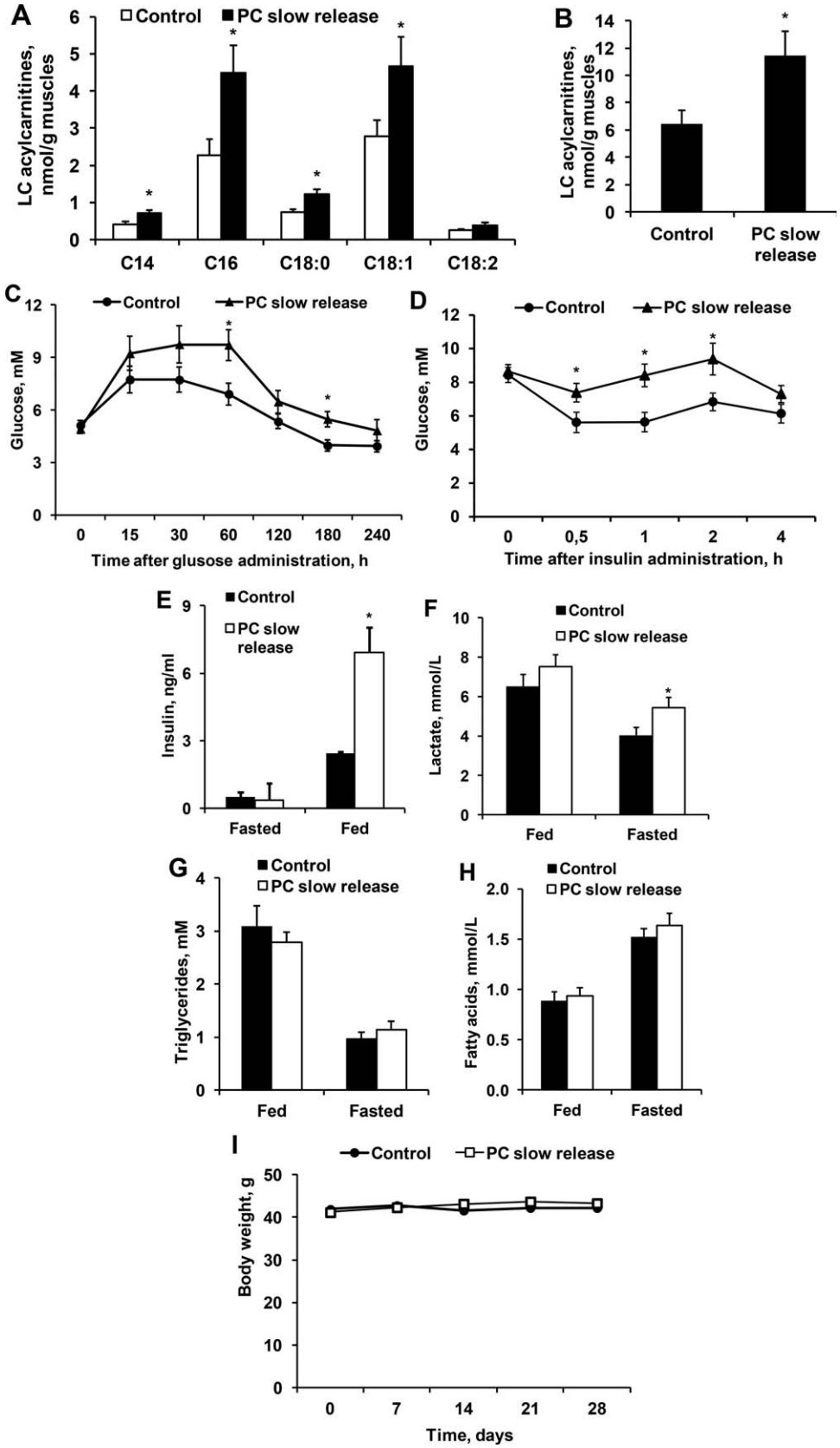

FIG 3

*Effects induced by the long-term, slow-release administration of palmitoylcarnitine. The slow-release administration of PC (10 mg/kg/day) for 28 days induced a marked increase in the content of all long-chain (LC) acylcarnitines (A) and the total content of LC acylcarnitines (B) in skeletal muscles in fed state. The changes in muscle acylcarnitine content induced disturbances in glucose tolerance (C) and reduced insulin sensitivity (D). PC induced a marked increase in insulin concentration in the fed state (E). The lactate concentration was increased in both the fed and fasted states (F), while PC did not influence triglyceride (G) and FA concentrations (H) and body weight (I). Each value represents the mean ± SEM of 10 animals. *Significantly different from the respective control group (Student's* t *test or ANOVA following Tukey's test,* $P < 0.05$*).*

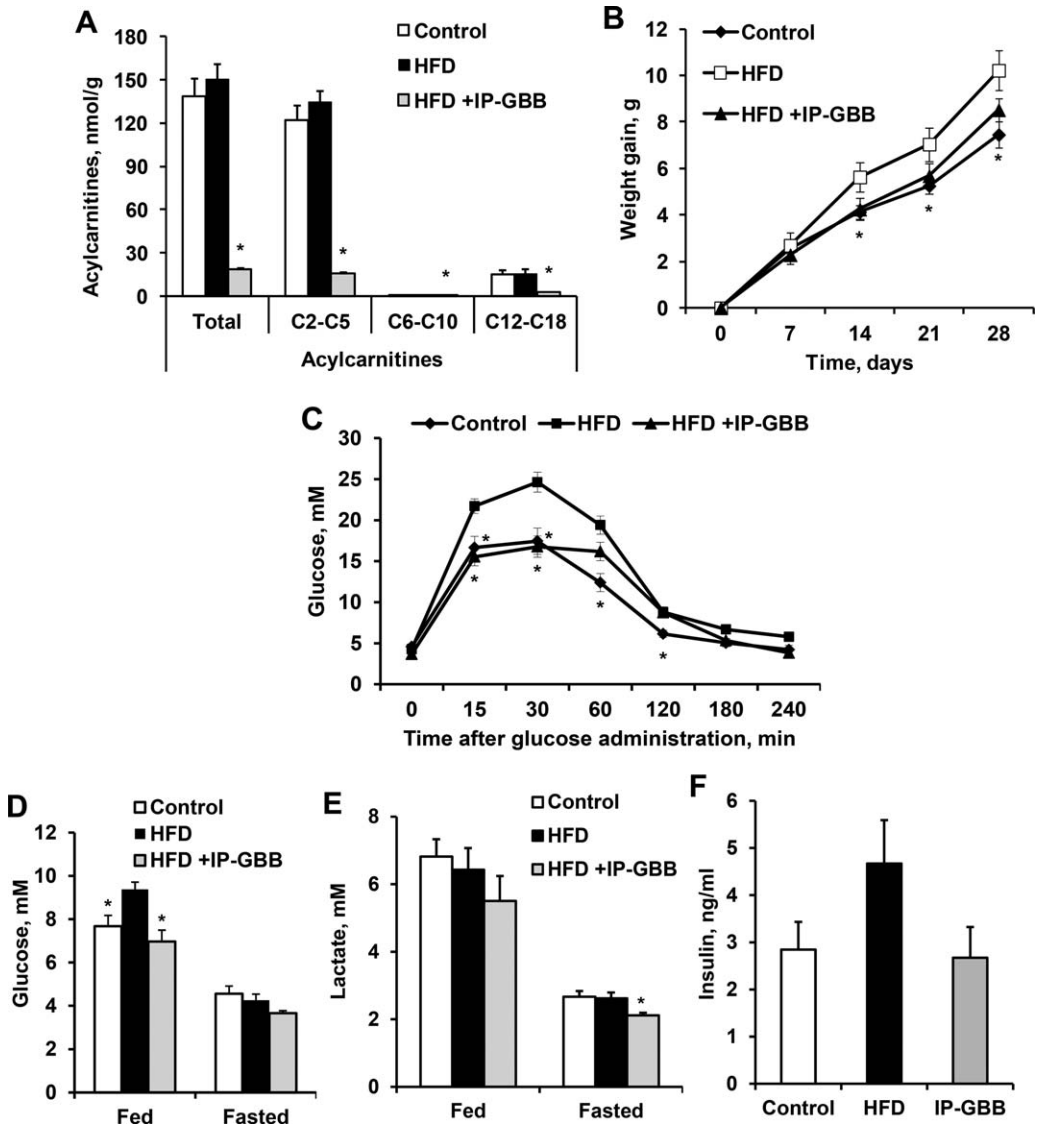

FIG 4

*Effect induced by the administration of the OCTN2 inhibitor IP-GBB in HFD-fed mice. IP-GBB at a dose of 10 mg/kg was administered for 28 days to HFD-fed mice. IP-GBB induced a significant decrease in acylcarnitine content in muscles (A). HFD feeding increased weight gain while IP-GBB did not significantly influenced weight gain (B). The administration of IP-GBB prevented HDF-induced impairments in glucose tolerance (C) and reduced the HFD-induced increase in fed state glucose concentration (D). The lactate (E) and insulin (F) concentrations in the fed state were not significantly increased in HFD-fed mice. Each value represents the mean ± SEM of 10 animals. *Significantly different from the HFD group (ANOVA following Tukey's test, $P < 0.05$).*

from 15.2 to 2.6 nmol/g. Similarly, IP-GBB treatment decreased the content of short- and medium-chain acylcarnitines.

The HFD induced significantly increased weight gain and marked glucose intolerance (Figs. 4B and 4C). In HFD-fed mice, the glucose concentrations at 15, 30, and 60 min after glucose administration were 5–7 mM higher compared to those of the control group. The long-term pharmacological decrease in acylcarnitine content prevented the HFD-induced disturbances in glucose tolerance (Fig. 4C). Thus, 15 and 30 min after glucose administration, the glucose concentrations in the IP-GBB-treated mice were similar to those of the control group. In addition, the decrease in acylcarnitine content reduced hyperglycemia in the fed state (Fig. 4D). Moreover, IP-GBB treatment stimulated complete glucose oxidation and reduced the lactate concentration in the fed and fasted states by 2.4 and 1.3 mM, respectively (Fig. 4E). IP-GBB treatment decreased the HFD-induced increase in insulin concentration in the fed state (Fig. 4F). Overall, although the content of LC acylcarnitines in HFD-fed mice was not increased, the pharmacological decrease of LC acylcarnitine content improved glucose tolerance.

4. Discussion

In this study, we show that LC acylcarnitines influence glucose metabolism *in vivo* and therefore are important players in the physiological regulation of energy metabolism. Moreover, the

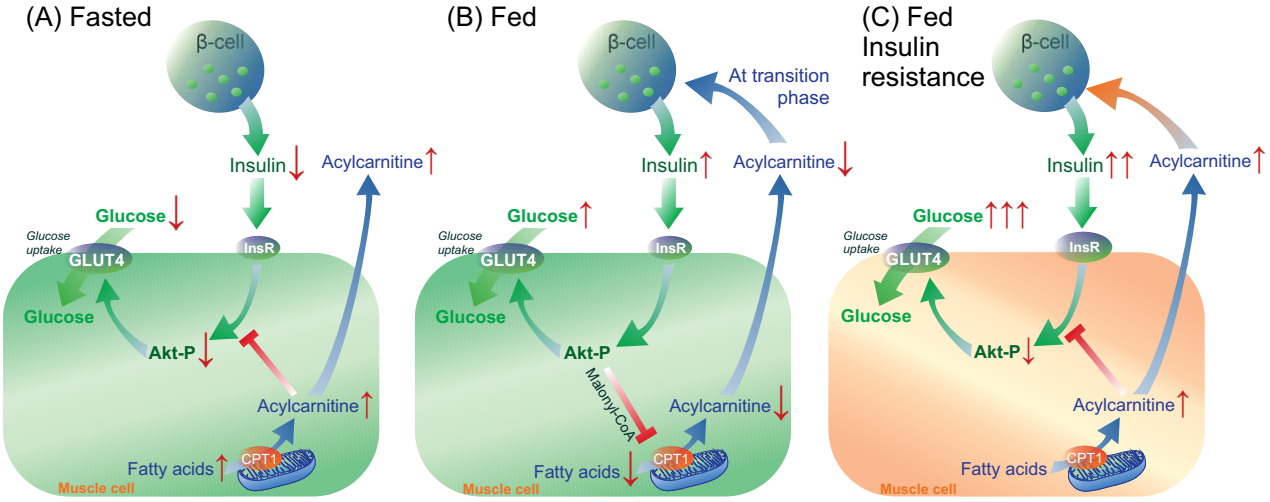

FIG 5

Physiological and pathological actions of long-chain acylcarnitines: implications for the development of insulin resistance. (A) In the fasted state, the low level of insulin is unable to inhibit acylcarnitine production, and the high acylcarnitine content continuously inhibits the phospho-Akt pathway and glucose uptake and metabolism. (B) Glucose in the fed state stimulates insulin release, which overcomes the acylcarnitine-induced effects on insulin signaling and reduces acylcarnitine production. Acylcarnitines in the presence of glucose facilitate insulin release to stimulate the transition from the fasted to fed state. (C) In the case of insulin resistance, insulin cannot effectively decrease acylcarnitine production in the fed state. This results in the accumulation of acylcarnitines, the continuous inhibition of Akt phosphorylation and subsequent disturbances in glucose uptake and metabolism. Thus, the accumulation of acylcarnitines during the fed state limits metabolic flexibility and accelerates hyperglycemia and hyperinsulinemia. The red arrows indicate whether the concentration is relatively high (↑) or low (↓).

accumulation of LC acylcarnitines during the fed state can accelerate diabetes progression by the simultaneous induction of insulin resistance and insulin release *in vivo*. A single-dose administration of PC in mice inhibits Akt phosphorylation in muscles and the downstream signaling pathways involved in glucose uptake. The long-term administration of PC induces insulin resistance, hyperinsulinemia and disturbances in glucose tolerance. In the model of lipid overload induced by a HFD, a decrease in LC acylcarnitine content restores glucose tolerance and insulin sensitivity and reduces hyperinsulinemia.

In previous studies, we hypothesized that LC acylcarnitines are not only markers for incomplete FA oxidation but also active FA metabolites involved in the regulation of energy metabolism [9,27]. In this study, we confirm this hypothesis through our results indicating that the acute and chronic administration of PC *in vivo* limits insulin signaling-induced effects and insulin-related glucose uptake in muscles. According to our results, the mechanism behind LC acylcarnitine action in muscles is the inhibition of Akt phosphorylation and the subsequent inhibition of downstream signaling. This is in line with previous studies in C2C12 myotubes [4,5]. Interestingly, an increased concentration of insulin can overcome the LC acylcarnitine-induced effects and stimulate Akt phosphorylation to the appropriate level. This explains how in the fed state in response to glucose, increased concentrations of insulin can overcome the inhibitory effects of high LC acylcarnitine content [8,28]. In addition, to support the transition from the fasted to fed state and overcome transient intramuscular insulin insensitivity, LC acylcarnitines facilitate insulin release.

In the fasted state, the low level of insulin is unable to inhibit LC acylcarnitine production, and the high LC acylcarnitine content continuously inhibits the Akt pathway. As a result, glucose uptake and metabolism is limited, while LC acylcarnitine synthesis by CPT-1 and subsequent FA oxidation increase. Therefore, in starved individuals, the intracellular content of LC acylcarnitines is higher than in the fasted state [28]. In our study, both a bolus and long-term administration of PC increased the content of C18 acylcarnitines, suggesting that a high LC acylcarnitine content blocks the insulin-induced CPT-1 inhibition and stimulates an even higher increase in LC acylcarnitine content. Many studies indicate that acylcarnitine accumulation is a result of incomplete FA oxidation [1,2,5,29]. Indeed, during ischemia or heart failure, acylcarnitines accumulate in mitochondria because of a transient or permanent inhibition of FA-dependent oxidative phosphorylation in mitochondria [10,30,31]. However, our results indicate that in certain conditions such as the fasted state, the physiologically important LC acylcarnitine accumulation is a result of their CPT-1-driven overproduction coupled to a high FA oxidation rate. Overall, LC acylcarnitines, as inhibitors of Akt phosphorylation, are active participants in an intercellular feedback mechanism of insulin signaling and are a substantial part of the energy metabolism regulation program (Figs. 5A and 5B).

In healthy subjects in the fed state, to inhibit FA metabolism and facilitate glucose metabolism the increased concentration of insulin inhibits LC acylcarnitine production via the increased tissue content of malonyl-CoA [1,28]. That occurs due to the insulin-induced inhibition of AMP-activated protein

kinase (AMPK) activity, which results in the stimulation of acetyl-CoA carboxylase (ACC; synthesis of malonyl-CoA) and inhibition of malonyl-CoA decarboxylase (MCD; degradation of malonyl-CoA) [32,33]. The inability of insulin to inhibit LC acylcarnitine production in the fed state induces disturbances in glucose uptake and metabolism. In the early stage of insulin resistance, hyperinsulinemia can compensate for insulin resistance and also overcome the LC acylcarnitine-induced effects. In the later stages of the disease, insulin resistance leads to the inability to inhibit LC acylcarnitine production and is accompanied by increased concentrations of LC acylcarnitines, which continuously inhibit the Akt-mediated signaling pathway and further stimulate the progression of glucose intolerance. Thus, the accumulation of LC acylcarnitines can accelerate the progression of insulin resistance (Fig. 5C).

Acylcarnitine accumulation has been detected in various models of advanced diabetes. Overall, it has been shown that the accumulation of LC acylcarnitines is not related to mitochondria dysfunction and usually reflects a higher FA oxidation rate in animal muscle and heart tissues [4,10,34,35]. In this study, at the early stage of insulin resistance and glucose intolerance induced by a high-fat diet, the muscle LC acylcarnitine content, similar to the free FA and blood glucose concentrations, was not significantly increased. This is an obvious effect of hyperinsulinemia, which at the early stages of insulin resistance can compensate for the inability of the muscle tissue to respond to lower insulin concentrations. At this stage of the disease, insulin resistance can be detected only by increased insulin concentrations and changes in the glucose and insulin tolerance or clamp tests. Similar to glucose and other markers, the LC acylcarnitine content is not markedly elevated at the early stages of insulin resistance but rather is a feature of severe diabetes.

The beneficial effects of genetically and pharmacologically induced LC acylcarnitine decreases have been demonstrated in models of insulin resistance. The limited production of acylcarnitines and oxidation of FA in $MCD^{-/-}$ knockout mice resulted in a remarkable protection against HFD-induced metabolic perturbations [4,34]. In addition, it was demonstrated that treatment with the CPT-1 inhibitor oxfenicine resulted in improved whole-body glucose tolerance and insulin sensitivity in the high-fat diet-induced insulin-resistance model [36]. Recently, a decrease in L-carnitine content was shown to decrease LC acylcarnitine content and in turn reduced circulating glucose and insulin concentrations [10]. The decrease in the LC acylcarnitine production has a more pronounced insulin-sensitizing effect at the early stage of insulin resistance when the LC acylcarnitine content is not increased. In contrast, a somewhat limited beneficial effect of the LC acylcarnitine decrease was observed in db/db mice, suggesting that the efficacy of this approach is limited in individuals with severely impaired insulin signaling either upstream or downstream of the Akt pathway. An important advantage of LC acylcarnitine-decreasing treatment is that it could be effectively combined with other treatments and lifestyle changes to achieve synergistic effects [10,37].

In this study, while the LC acylcarnitine content was not increased in HFD-treated mice, the pharmacologically induced decrease of LC acylcarnitines markedly improved glucose tolerance. The labeled [^{3}H]-DOG *in vivo* uptake results (data not shown) suggest that a 4-week HFD treatment initially induces insulin resistance in adipose tissue but not in muscles. Therefore, muscle LC acylcarnitine accumulation in the fed state of HFD-fed mice was not observed. Nevertheless, a pharmacological-mediated decrease in LC acylcarnitine content improved muscle insulin sensitivity, which compensated for the adipose tissue insulin resistance and thus improved the overall glucose tolerance and prevented fed-state hyperinsulinemia. This confirms that LC acylcarnitines at physiological concentrations are regulators of energy metabolism and that by decreasing the physiological levels of LC acylcarnitines, it is possible to improve muscle insulin sensitivity.

In conclusion, taking into account the importance of muscle metabolic flexibility and the ability to switch between fed and fasted states, LC acylcarnitines play a role in the regulation of energy metabolism. Our results link the development of skeletal muscle insulin resistance to the excessive accumulation of LC acylcarnitines and, moreover, suggest that acylcarnitines could induce insulin resistance independently of FA content and oxidation rate. These findings suggest that acylcarnitine decrease could be an effective pharmacologic strategy to prevent the acceleration of diabetes.

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CONFLICT OF INTEREST

There is no conflict of interest.

AUTHOR CONTRIBUTIONS

E.L., M.M.-K., and M.D. designed the research. M.M.-K., E.M., K.V., K.Vi., U.A., J.K., and R.V. conducted experiments. E.S., D.L., E.Lo., and S.G. performed organic synthesis and analytical chemistry. E.L., M.M-K., E.M., and M.D. analyzed and interpreted the data. E.L. wrote the manuscript. The study was supervised by E.L. and M.D. All authors read and approved the final manuscript.

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