Original Article

Retention of acetylcarnitine in chronic kidney disease causes insulin resistance in skeletal muscle

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Insulin resistance occurs frequently in patients with chronic kidney disease. However, the mechanisms of insulin resistance associated with chronic kidney disease are unclear. It is known that an increase in the mitochondrial acetyl-CoA (AcCoA)/CoA ratio causes insulin resistance in skeletal muscle, and this ratio is regulated by carnitine acetyltransferase that exchanges acetyl moiety between CoA and carnitine. Because excess acetyl moiety of AcCoA is excreted in urine as acetylcarnitine, we hypothesized that retention of acetylcarnitine might be a cause of insulin resistance in chronic kidney disease patients. Serum acetylcarnitine concentrations were measured in chronic kidney disease patients, and were significantly increased with reduction of renal function. The effects of excess extracellular acetylcarnitine on insulin resistance were studied in cultured skeletal muscle cells (C2C12 and human myotubes), and insulin-dependent glucose uptake was significantly and dose-dependently inhibited by addition of acetylcarnitine. The added acetylcarnitine was converted to carnitine via reverse carnitine acetyltransferase reaction, and thus the AcCoA concentration and AcCoA/CoA ratio in mitochondria were significantly elevated. The results suggest that increased serum acetylcarnitine in CKD patients causes AcCoA accumulation in mitochondria by stimulating reverse carnitine acetyltransferase reaction, which leads to insulin resistance in skeletal muscle.

***Key Words*: chronic kidney disease, insulin resistance, acetylcarnitine, skeletal muscle, mitochondria**

Chronic kidney disease (CKD) is frequently accompanied by insulin resistance as a clinical abnormality.[1] Insulin resistance develops from the early stage of renal failure in non-diabetic CKD patients[2] and aggravates along with reduction of renal function.[3] Many factors have been proposed as causes of insulin resistance in CKD, but the main mechanisms are unclear.

In uremic patients, DeFronzo *et al.*[4] showed insulin resistance in skeletal muscle in the lower limb using the euglycemic insulin clamp technique. Friedman *et al.*[5] demonstrated insulin resistance in skeletal muscle, but not in liver, of uremic patients using an intravenous glucose tolerance test and the hyperinsulinemic euglycemic clamp technique. These results led to the suggestion that any alternative steps in insulin-dependent glucose transport process, rather than a defect in insulin receptor function or depletion of glucose transporter 4 (GLUT4), is involved in insulin resistance in skeletal muscle.[5] Glucose uptake via GLUT4 in cultured adipocytes isolated from normal rat is significantly inhibited after treatment with sera from non-hemodialyzed and non-diabetic CKD patients,[6] and inhibition is significantly reduced by addition of hemodialyzed sera to the cells.[7] These results suggest that sera from CKD patients contain compounds that induce insulin resistance in skeletal muscles.

Carnitine (CT) is a low-molecular-weight compound that is synthesized from lysine and methionine and is abundant in skeletal muscles.[8,9] CT has an essential role as a carrier of long chain fatty acids through the mitochondrial inner membrane for β-oxidation (Fig. 1). Reaction of CT with the acyl group of acyl-CoA forms acyl-CT, which is translocated to the mitochondrial matrix[8] and then reconverted to acyl-CoA and β-oxidized to acetyl-CoA (AcCoA) for energy production. Mitochondrial AcCoA is the initial source for aerobic energy production and is also an allosteric inhibitor of pyruvate dehydrogenase (PDH), an enzyme that converts pyruvate to AcCoA (Fig. 1).[10] Thus, if the level of mitochondrial AcCoA exceeds the reactive capacity of the TCA cycle, the glycolytic pathway from pyruvate to AcCoA is inhibited by negative feedback, which may lead to insulin resistance.[10] However, another important mechanism restores excess mitochondrial AcCoA to CoA through conversion of CT to acetylcarnitine (AcCT) by mitochondrial carnitine acetyltransferase (CrAT) (Fig. 1).[11,12] Recently, Muoio *et al.*[13] showed that production of AcCT by CrAT and smooth efflux of AcCT from mitochondria to extracellular fluids was a crucial mechanism for prevention of insulin resistance.

Since AcCT excreted from skeletal muscle to blood is finally eliminated in urine,[14] blood AcCT may be increased in CKD patients. Fouque *et al.*[15] showed that serum concentrations of acylated CT (acyl-CT) were significantly higher in CKD patients than in normal subjects. Total blood CT consists of free CT and acyl-CT,[16] and most blood acyl-CT comprises AcCT.[17] If blood AcCT in CKD patients is indeed elevated, CrAT activity must be inhibited in skeletal muscles because of the stimulation of the reverse direction of this enzyme reaction. To examine this hypothesis, we measured serum AcCT levels in CKD patients and studied the effects of exogenous AcCT on insulin-dependent glucose uptake and the concentration of mitochondrial AcCoA in cultured skeletal muscle cells. Our results suggest that retention of AcCT is one of the reasons for insulin resistance in CKD patients.

Materials and Methods

Materials. Acetyl-L-carnitine HCl was purchased from Tokyo Kasei Kogyo (Tokyo, Japan), and acetyl-L-[$^2H_3$]carnitine (AcCT-d3) HCl, L-[$^2H_3$]carnitine (CT-d3) HCl, and DL-[$^2H_9$]carnitine (CT-d9) HCl were obtained from C/D/N Isotopes Inc. (Quebec, Canada). Acetyl-DL-[$^2H_9$] carnitine (AcCT-d9) was synthesized from CT-d9 by reaction with acetic anhydride and pyridine. Briefly, a solution of carnitine-d9 (3.3 mg in 10 μl H_2O) was added to 100 μl of pyridine. After vortex mixing, 1 ml of acetic anhydride was added to the solution and mixed using a magnetic stirrer overnight at 4°C. The reactant was dried at 80°C with N_2 and

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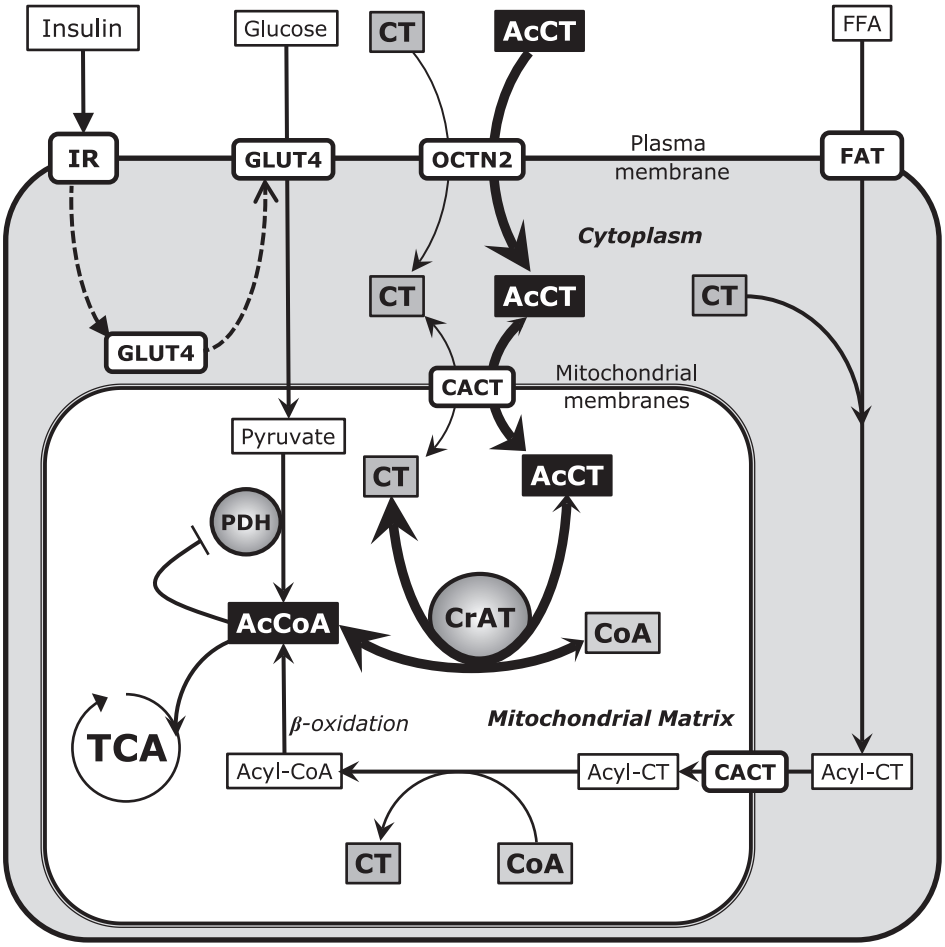

Fig. 1. Mitochondrial reversible reaction between AcCT and CT and between CoA and AcCoA in skeletal muscles. Mitochondrial AcCoA is produced from the conversion of acetyl-group of AcCT or from acyl-CoA in β-oxidation. Exogenous AcCT is transported from extracellular fluids in cell and mitochondria through OCTN2 and CACT, respectively. The excess acetyl group of AcCT can be transferred to free CoA by the reverse CrAT reaction in mitochondria, with consequent production of AcCoA and free CT. On the other hand, in β-oxidation, excess acetyl groups of AcCoA are transferred to CT by the forward CrAT reaction, with formation of free CoA and AcCT. Excess accumulation of AcCoA in mitochondria would cause stagnation of glycolytic pathway through allosteric inhibition of PDH. Abbreviations: AcCoA, acetyl-CoA; AcCT, acetylcarnitine; Acyl-CT, acylcarnitine; CACT, carnitine acylcarnitine translocase; CrAT, carnitine acetyltransferase; CoA, coenzyme A; CT, carnitine; FFA, free fatty acid; GLUT 4, glucose transporter 4; IR, insulin receptor; OCTN2, organic anion transporter type-2; PDH, pyruvate dehydrogenase; TCA, tricarboxylic acid.

the residue was dissolved in 100 µl of 0.1% HCOOH/H_2O solution. The synthesized AcCT-d9 was characterized by liquid chromatography-tandem mass spectrometry (LC-MS/MS). IPC-DBAA (0.5 M dibutylammonium acetate in water) was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). L-Carnitine and additional reagents and solvents were purchased from Wako Pure Chem. Ind. (Osaka, Japan) and AcCoA, propionyl CoA and CoA were from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified eagle medium (DMEM) containing 50 mM pyruvate, 50 mM glutamate, 1% streptomycin, 1% penicillinand 1% amphotericin B was used in all cell culture experiments. The reagents were purchased from Thermo Fischer Scientific Inc. (Gibco®, Waltham, MA).

Serum CT and AcCT concentrations in the patients with kidney diseases. Serum CT and AcCT concentrations were determined in 75 patients with CKD diagnosed along the KDIGO Clinical Practice Guideline for the Evaluation and Management for Clinic Kidney Disease[18] (42 males and 33 females; age 62.5 ± 1.6 years, mean ± SEM). The causes of CKD were chronic glomerulonephritis ($n = 36$), IgA nephropathy ($n = 15$), hypertension ($n = 8$), lupus nephritis ($n = 5$), membranous nephropathy ($n = 4$), autosomal dominant polycystic kidney disease ($n = 3$), ANCA-associated vasculitis ($n = 2$), focal glomerulosclerosis ($n = 1$) and tubulointerstitial nephropathy ($n = 1$). Glomerular filtration rate was estimated by modified MDRD equation adapted for the Japanese population: eGFR = 194 × (serum creatinine in mg/dl)$^{-1.094}$ × age$^{-0.287}$ × (0.739 for women).[19] Patients with hemodialysis or diabetes mellitus were excluded in this study. Serum collected from the patients in the morning was stored at −20°C until analysis. Informed consent was obtained from all patients. The study protocol was approved by the Ethics Committee of Tokyo Medical University Ibaraki Medical Center.

Carnitine and AcCT concentrations in serum were quantified by LC-MS/MS using a modified method based on that in Ghoshal *et al.*[20] Serum (5 µl) was mixed with 5 ng of AcCT-d3 and CT-d3 as internal standards in 100 µl of acetonitrile-water (19:1, *v/v*). After centrifugation at 2,000 × *g* for 1 min, the liquid phase was evaporated at 80°C under a nitrogen stream. The residue was redissolved in 80 µl of 0.1% HCOOH in water, and an aliquot (1 µl) was analyzed by LC-MS/MS in electrospray ionization (ESI) mode. Chromatographic separation was performed using a Hypersil GOLD aQ column (150 × 2.1 mm, 3 µm, Thermo Fischer Scientific Inc.) at 40°C. The mobile phase was methanol-water (1:9, *v/v*) containing 0.1% formic acid, at a flow rate of

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200 μl/min. The MS/MS conditions were spray voltage, 3,000 V; vaporizer temperature, 450°C; sheath gas (nitrogen) pressure, 50 psi; auxiliary gas (nitrogen) flow, 15 arbitrary units; ion transfer capillary temperature, 220°C; collision gas (argon) pressure, 1.0 motor; collision energy, 20 V; ion polarity, positive; and selected reaction monitoring (SRM), *m/z* 162 → *m/z* 103, *m/z* 165 → *m/z* 103, and *m/z* 171 → *m/z* 103 for CT, [$^2H_3$] variant, and [$^2H_9$] variant, respectively, and *m/z* 204 → *m/z* 85, *m/z* 207 → *m/z* 85, and *m/z* 213 → *m/z* 85 for AcCT, [$^2H_3$] variant and [$^2H_9$] variant, respectively.

The effect of endogenous AcCT on insulin-dependent 2DG uptake in cultured mouse C2C12 and human primary myotubes. Mouse differentiable myoblasts (C2C12; ATCC, Manassas, VA) were cultured with growth medium (DMEM with 10% fetal bovine serum) until confluent, and then the medium was replaced with differentiation medium (DMEM with 2% horse serum). In addition, normal human primary skeletal muscle myoblast cell isolated from the rectus abdominis muscle (ZenBio, Inc., NC) were used in the present study. The human myoblasts were cultured until confluent and differentiated to myotubes with dedicated growth and differentiation media (ZenBio, Inc.) recommended by manufactural instruction along with our previous method.[21] After 5 days, the differentiated myotubes were used in experiments. Cells were maintained at 37°C in a humid atmosphere of 5% CO_2 in air.

The effects of exogenous AcCT on glucose uptake in the C2C12 and human myotubes were evaluated using a 2-deoxyglucose (2DG) uptake kit (Cosmo Bio Co., Ltd, Tokyo, Japan). Differentiated C2C12 and human myotubes grown in a 6-well and 12-well plates, respectively, were treated with 0, 50, 100 or 300 μM AcCT in differentiation medium for 24 h. Thereafter, cells were washed with PBS and pre-incubated with serum-free DMEM medium containing 0, 50, 100 or 300 μM AcCT for 6 h. After the pre-incubation, cells were washed with PBS three times, exposed to 1 μM insulin with PBS including 2% BSA for 18 min, and then 1 mM 2DG (final concentration) was added to the medium. After 20 min, the cells were washed with PBS three times, collected in 1 ml of 10 mM Tris-HCl (pH 8.0), and homogenized. The resulting sample was divided into two aliquots for 2DG uptake and protein assays. One aliquot was heated at 80°C for 15 min and centrifuged at 15,000 × *g* for 20 min. The supernatant was used for the 2DG uptake assay. The other aliquot was used for determination of total protein content by Lowry's method[22] using a Pierce® BCA protein assay kit (Thermo Fischer Scientific Inc.). The 2DG concentration is shown per total protein.

Kinetics of exogenous AcCT in C2C12 myotubes. The differentiated C2C12 myotubes grown in a 100-mm dish were also exposed to growth medium containing various concentrations of authentic AcCT (0, 50, 100 and 300 μM) with or without 50 μM of deuterium-labeled CT (d3). After 24 h, the cells were harvested in KP buffer (10 mM potassium-phosphate buffer, pH 5.5), and mitochondrial and cytosolic fractions were prepared along with the previous method.[23] Collected myotubes were homogenized with a loose-fitting Teflon pestle in 4 volumes of 3 mM Tris-HCl buffer (pH 7.4) containing 0.25 mM sucrose and 0.1 mM EDTA. The homogenate was centrifuged at 700 × *g* for 10 min and the supernatant was centrifuged again at 7,000 × *g* for 20 min. The pellet was homogenized with 100 μl KP buffer and used as the mitochondrial fraction. The supernatant from 7,000 × *g* was further centrifuged at 105,000 × *g* for 90 min and the final supernatant was used as the cytosolic fraction. After addition of AcCT-d9 and CT-d9 as internal standards to each fraction, unlabeled and d3-labeled forms of CT and AcCT in the mitochondrial or cytosolic samples (50 μl) were quantified by LC-MS/MS. The mitochondrial and cytosolic concentrations of CT and AcCT were calculated per mg protein in each fraction.

Mitochondrial CoA and AcCoA concentrations in C2C12 myotubes. C2C12 myotubes grown in a 100-mm dish were exposed to growth medium containing various concentrations of AcCT (0, 50, 100 and 300 μM) for 24 h. Thereafter, mitochondrial pellet was collected by the method as described above. For AcCoA and CoA quantifications, the collected mitochondria were prepared by Agnieszka's method with minor modifications.[24] Mitochondrial pellet was homogenized in 100 μl of freshly prepared 100 mM KH_2PO_4 (pH 4.9) and 100 μl of acetonitrile, 2-propanol and methanol (3:1:1, *v/v/v*) with 50 pmol of the propionyl CoA as an internal standard, and then, centrifuged at 16,000 × *g* at 4°C for 10 min. The supernatant was collected, and the pellet was re-extracted again with the 100 μl of acetonitrile, 2-propanol and methanol (3:1:1, *v/v/v*). After second centrifugation, two supernatants were combined and dried at 50°C under nitrogen stream. The dried extract was re-suspended in 50 μl of 50% methanol-water solution and centrifuged at 14,000 × *g* for 10 min at 4°C. This supernatant was used for quantification of mitochondrial AcCoA and CoA. Five μl of supernatant after the final centrifugation was injected into a LC-MS/MS system and analyzed in ESI mode. Chromatographic separation was performed using a Hypersil GOLD aQ column (150 × 2.1 mm, 3 μm) at 40°C, with the following gradient system at a flow rate of 200 μl/min. Initially, the mobile phase comprised 5 mM aqueous dibutylammonium acetate (DBAA)-methanol (4:1, *v/v*), which was programmed to change to 5 mM aqueous DBAA-methanol (3:7, *v/v*) in a linear manner over 10 min. The MS/MS conditions were spray voltage, 2,500 V; vaporizer temperature, 450°C; sheath gas (nitrogen) pressure, 50 psi; auxiliary gas (nitrogen) flow, 15 arbitrary units; ion transfer capillary temperature, 220°C; collision gas (argon) pressure, 1.0 motor; collision energy, 35 V; ion polarity, negative; and SRM, *m/z* 766 → *m/z* 408, *m/z* 808 → *m/z* 408, and *m/z* 822 → *m/z* 408 for CoA, AcCoA, and propionyl-CoA, respectively. Mitochondrial concentrations of CoA and AcCoA were calculated per mg protein.

Mitochondrial reverse CrAT activity in C2C12 myotubes. Reverse CrAT activity was measured using C2C12 myotubes. Naïve mitochondrial pellet isolated from the cells was re-suspended in CelLytic lysis buffer (Sigma Aldrich) and sonicated at five times one second pulses on ice. Two hundred μg of the mitochondrial lysate was warmed at 37°C for 5 min, and then was added to pre-incubated (37°C, 5 min) 50 mM Tris-HCl buffer (pH 7.8) containing 500 μM AcCT, 500 μM CoA and 1 mM EDTA.[25] The mixture (total volume of 200 μl) was incubated at 37°C for 0, 5, 10, 15 and 20 min, and AcCoA and CT were quantified by LC-MS/MS. Productions of AcCoA and CT were calculated per mg mitochondrial protein.

The effect of exogenous AcCT treatment on cell viability in myotubes. Cell viability was evaluated by MTT assay.[26] Differentiated C2C12 and human myotubes were exposed to 0–300 μM AcCT for 24 h and then incubated with 0.5 mg/ml MTT in medium for 4 h at 37°C. The blue Formosan compound was solubilized in methanol containing 0.01 N HCl. Absorbance was determined spectrophotometrically at 560 nm, with 670 nm used as a reference.

Statistical analyses. Data are shown as means ± SEM or 2 × SEM. Significant differences were evaluated by one-way ANOVA with a post-hoc Dunnett multiple comparison test or by paired Student's *t* test using SPSS software (IBM Corp., Tokyo, Japan). $P<0.05$ was considered significant. Correlations between eGFR value and serum concentration of AcCT or CT were analyzed by Pearson's correlation coefficient.

Results

Serum free CT and AcCT concentrations in patients with kidney diseases. Serum free CT concentrations in CKD patients were distributed without association with eGFR (Fig. 2A). In contrast, a significant eGFR-related increase in the serum AcCT concentrations was observed (Fig. 2B). Particularly, higher levels

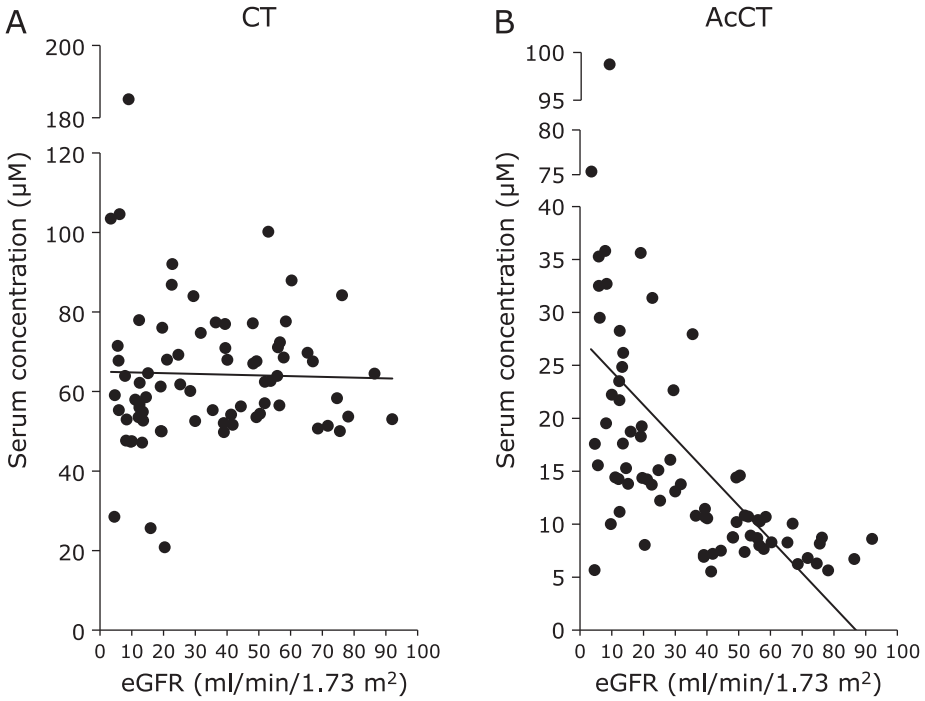

Fig. 2. Correlation relationship between eGFR value and serum CT or AcCT concentrations in CKD patients (n = 75). (A) CT, carnitine; r^2 = 0.00461, p = 0.857; (B) AcCT, acetyl carnitine; r^2 = 0.27074, p<0.0001. Abbreviations: CKD, chronic kidney disease; CT, carnitine; AcCT, acetylcarnitine; eGFR, estimated glomerular filtration rate.

of serum AcCT were observed in CKD patients with eGFR <35 ml/min/1.73 m^2, and the highest concentration reached approximately 100 μM. In each disease, there were significant negative correlation between serum AcCT concentration and eGFR value in chronic glomerulonephritis (n = 36; r^2 = 0.2233, p<0.01) and IgA nephropathy (n = 15; r^2 = 0.5793, p<0.001), while no correlation was observed between serum CT and eGFR in both diseases. Significant relationships between serum AcCT concentration and eGFR were not detected in other diseases because of fewer numbers of patients.

Effects of AcCT on 2DG uptake by myotubes. Insulin-dependent uptake of 2DG by C2C12 and human myotubes was dose-dependently inhibited by exogenous AcCT (Fig. 3A and B), which implies that an increased concentration of extracellular AcCT causes insulin resistance in skeletal muscle. AcCT had no significant cytotoxic effect on both myotubes in MTT assays of cell viability at concentrations of 0–300 μM (Fig. 3C and D).

Effects of AcCT on mitochondrial and cytosolic levels of CT and AcCT in myotubes. Mitochondrial and cytosolic concentrations of CT and AcCT in myotubes treated with exogenous AcCT and CT were examined using authentic and deuterium-labeled compounds to distinguish the origins. C2C12 cells were treated with 0–300 μM of authentic AcCT (AcCT-d0) and 50 μM of CT-d3 for 24 h. The concentrations of unlabeled and d3-labeled CT and AcCT were measured in mitochondrial and cytosolic fractions isolated from the C2C12 myotubes (Fig. 4). With addition of 50 μM CT-d3 alone to the medium, approximately one-fourth and one-eighth of the CT-d3 was converted to AcCT-d3 in mitochondria and cytosol, respectively (Fig. 4A). However, when 50 μM of CT-d3 was added with increasing amounts of AcCT-d0, both uptake of CT-d3 and conversion to AcCT-d3 decreased in a dose-dependent manner. AcCT-d0 and authentic CT (CT-d0) that appeared to be produced from AcCT-d0 in mitochondria were significantly increased, along with increasing concentrations of AcCT-d0 in media, and the mitochondrial level of CT-d0 was approximately 2- to 3-fold higher than that of AcCT-d0. In the cytosolic fraction, the kinetics of CT and AcCT were virtually the same as those in the mitochondria (Fig. 4B). Although CrAT is mainly expressed in mitochondria,[27] significant amounts of CT-d0 and AcCT-d3 were detected in the cytosolic fraction. This result suggests that these CrAT metabolites are preferably excreted from mitochondria into cytosol.

Effects of AcCT on the mitochondrial AcCoA and CoA concentrations in myotubes. Fig. 5 shows mitochondrial concentrations of AcCoA and CoA in myotubes after treatment with AcCT. After exposing to various doses of AcCT for 24 h, mitochondrial AcCoA concentrations in myotubes were dose-dependently increased while mitochondrial free CoA concentrations did not change significantly (Fig. 5A). Consequently, mitochondrial AcCoA/CoA ratios were also increased in a dose-dependent manner (Fig. 5B).

Reverse CrAT activity in mitochondria. Mitochondrial reverse CrAT activity was determined in naïve mitochondria isolated from C2C12 myotubes (Fig. 6). When the mitochondria were incubated with AcCT and free CoA, AcCoA (Fig. 6A) and free CT (Fig. 6B) were produced time-dependently up to 20 min. The enzyme activities calculated by AcCoA and CT productions for 5 min were 13.0 and 9.9 nmol/min/mg protein, respectively. These results confirm that excess AcCT is converted to AcCoA in mitochondria by the reverse CrAT activity.

Discussion

The mechanisms of insulin resistance in CKD patients were unclear. The results of this study showed that serum AcCT concentrations in CKD patients were increased with reduction of renal function evaluated by eGFR. Cell culture experiments showed that exogenous AcCT caused insulin resistance in skeletal muscle cells in a dose-dependent manner. The added AcCT was converted to CT in the cells, along with elevation of the mitochondrial AcCoA/CoA ratio. These findings suggest that disturbance of glucose metabolism in skeletal muscle of CKD patients is

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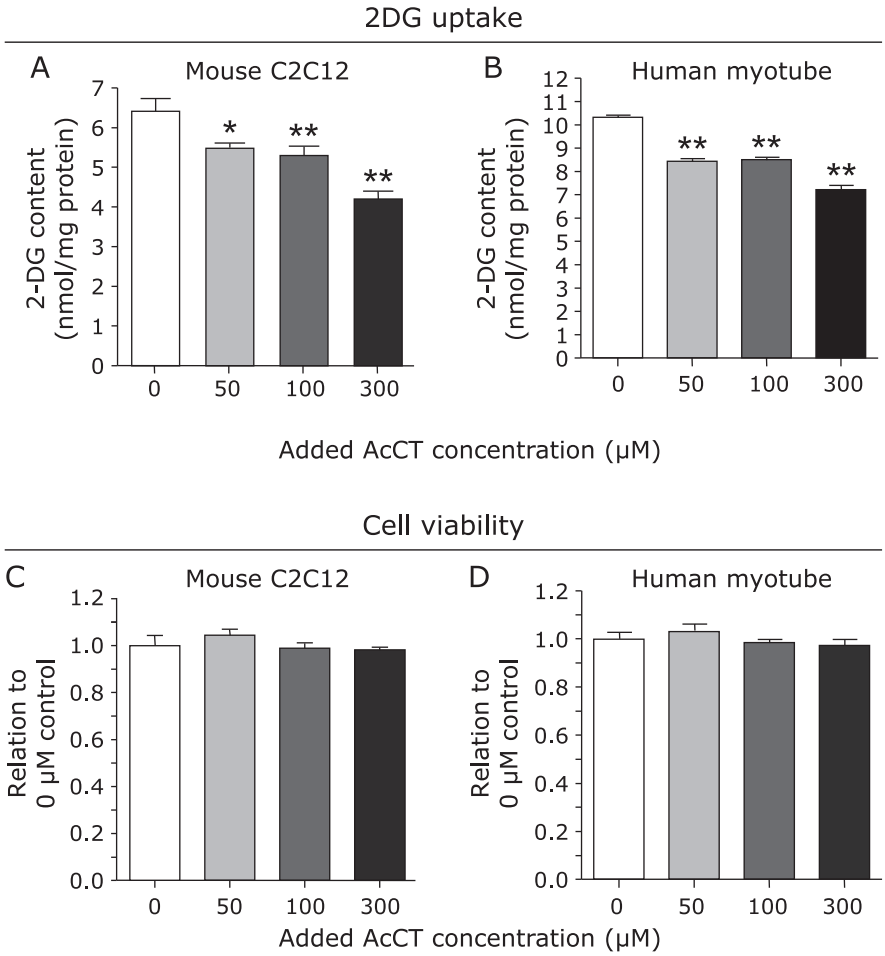


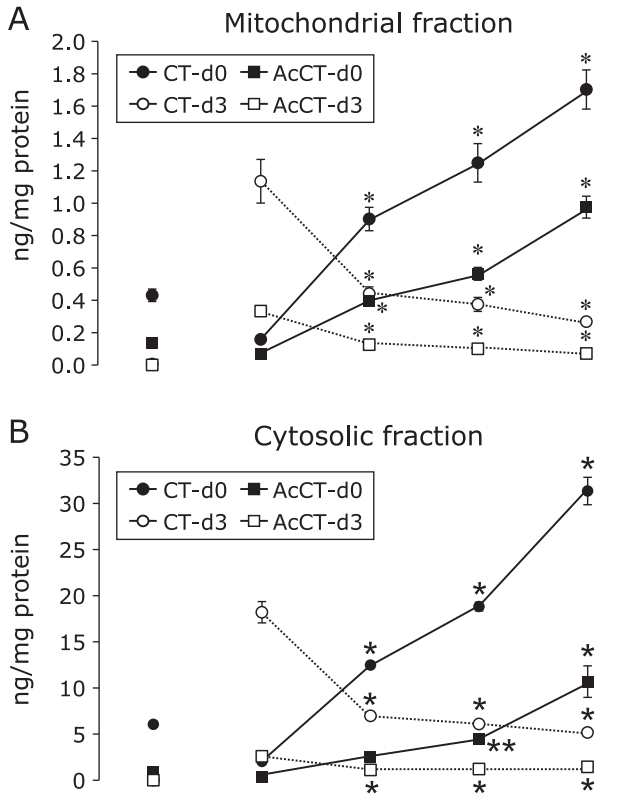
Fig. 3. Effects of exogenous AcCT on 2-DG uptake and cell viability in mouse C2C12 and human myotube cells. Differentiated C2C12 and human myotubes were exposed to AcCT (0–300 μM) for 24 h. Thereafter, insulin-dependent glucose uptake in C2C12 (A), human myotube (B), cell viability in C2C12 (C) and human myotube (D) were evaluated using a 2-DG uptake assay kit and an MTT assay, respectively. Data are expressed as the mean ± SEM in seven (A, B) or four (C, D) independent assays. (A, B) $p<0.0001$ for 2-DG uptake. (C, D) No significant difference for cell viability. *$p<0.05$, **$p<0.01$ vs 0 μM control (one-way ANOVA). Abbreviation: 2DG, 2-deoxyglucose.

caused by increased blood AcCT and elevation of the AcCoA/CoA ratio in mitochondria.

The mitochondrial enzyme CrAT converts AcCoA into CoA by transfer of the acetyl group to form membrane-permeable AcCT, thus maintaining the mitochondrial AcCoA/CoA ratio (Fig. 1).[11,12] Our data demonstrated that CrAT transfer of an acetyl group between CoA and CT was reversible and the acetyl group of AcCT was transferred to CoA in the presence of excess AcCT in muscle mitochondria (Fig. 6). In the cell culture experiment using stable isotopes, the concentration of CT derived from exogenous AcCT was significantly increased in both mitochondrial and cytosolic fractions (Fig. 4). In addition, mitochondrial AcCoA level was significantly increased in the myotubes when the cells were exposed to AcCT (Fig. 5). All results support our idea that the excess AcCT was converted to CT together with the production of AcCoA from free CoA by reverse CrAT reaction.

CrAT localizes predominantly in the mitochondrial matrix, although some activity may be detected in peroxisomes and the endoplasmic reticulum.[27] In the current study, the kinetics of CT and AcCT in mitochondrial and cytosolic fractions were similar (Fig. 4). These results strongly suggest that conversion of added AcCT to CT occurred in mitochondria, and that the CT produced was easily excreted from mitochondria to cytosol. It should be noted that both CT and AcCT concentrations were higher in the cytosolic fraction than in mitochondria. In normal rat heart tissue, Idyll-Wenger *et al.*[28] found that mitochondria contained only 8–9% of total cellular CT, while approximately 95% of cellular CoA was in mitochondria.

Mitochondrial accumulation of AcCoA affects glucose metabolism in skeletal muscle. Randle *et al.*[10] proposed the glucose-fatty acid cycle theory, in which byproducts of fatty acid β-oxidation (i.e., AcCoA, NADH and ATP) cause negative-feedback inhibition of the PDH complex, and consequently lead to insulin resistance. PDH catalyzes an irreversible reaction from pyruvate to AcCoA, and this activity is inhibited through phosphorylation of the E1 component by pyruvate dehydrogenase kinase (PDK).[29,30] Gene expression of PDK4, the main isoform of PDK in skeletal muscles, is upregulated by mitochondrial AcCoA accumulation.[31–33] In a muscle-specific CrAT knockout mouse, Muoio *et al.*[13] showed that transfer of acetyl groups from excess AcCoA to form AcCT by normal CrAT activity and subsequent AcCT efflux from mitochondria prevented reduction of PDH activity when fatty acid β-oxidation was activated in skeletal muscle. The reduction of PDH activity induces a delayed flux through oxidative reactions of glucose metabolism[34] and also results in accumulation of glucose-6-phosphate due to reduced phosphofructokinase activity regulated by the cellular energy state. Accumulation of glucose-6-phosphate allosterically reduces



AcCT-d0 (μM)	0	0	50	100	300
CT-d3 (μM)	0	50	50	50	50

Fig. 4. Mitochondrial and cytosolic concentrations of CT and AcCT in C2C12 myotubes exposed to various concentrations of AcCT with 50 μM CT-d3 for 24 h. Authentic and d3 variants of CT and AcCT were measured in mitochondrial (A) and cytosolic (B) fractions. Origins of CT-d0 and AcCT-d3 are added to AcCT-d0 and CT-d3, respectively. The table below the figure shows the added concentrations of AcCT-d0 and CT-d3. CT and AcCT levels in the mitochondrial and cytosolic fractions were calculated per mg protein. Data are shown as the mean ± SEM obtained in quadruplicate assays. $p<0.001$ (one-way ANOVA) in all comparisons. $^{**}p<0.05$, $^{*}p<0.01$ vs the respective 0 μM AcCT/50 μM CT-d3 control.

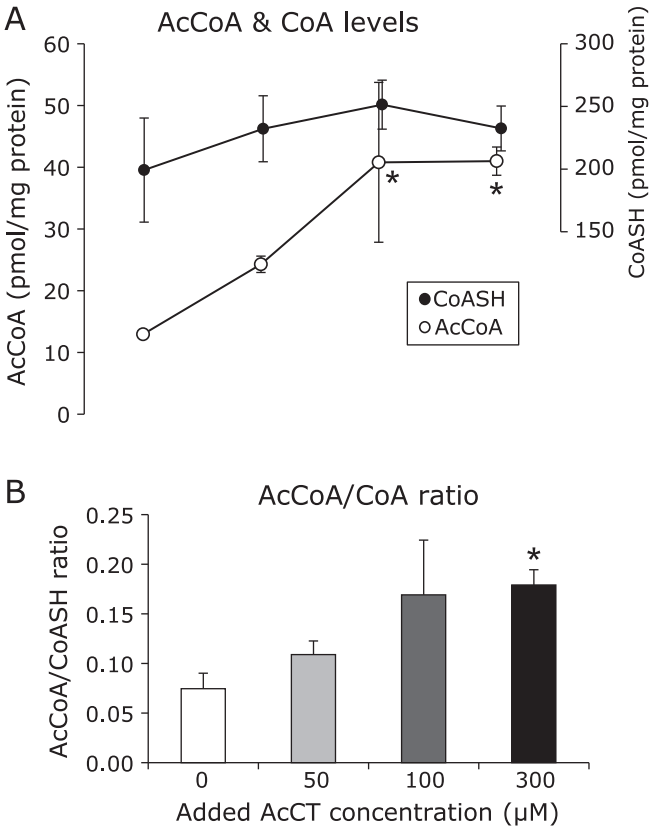


Fig. 5. Mitochondrial AcCoA and CoA concentrations (A) and the ratio of AcCoA to CoA (B) in C2C12 myotubes exposed to AcCT (0–300 μM) for 24 h. Mitochondrial AcCoA and CoA concentrations were calculated per mg mitochondrial protein. Data are shown as the mean ± SEM obtained in a quadruplicate assay. $^{*}p<0.05$ (one-way ANOVA) vs the respective 0 μM AcCT condition.

the activity of hexokinase, a rate-limiting enzyme, and finally causes insulin resistance.[35–38]

In the current study, increased serum AcCT was observed with decreased renal function in CKD patients. In contrast, CT was not significantly affected by the CKD stage. Because the significant negative correlations between serum AcCT concentration and eGFR value were observed in both chronic glomerulonephritis and IgA nephropathy, the increased serum AcCT level would depend on renal dysfunction rather than the causes of renal diseases. Both CT and AcCT are excreted in urine, but the renal clearance of acyl-CT is 4–8 fold higher than that of free CT.[39] Therefore, urinary clearance of AcCT is likely to be more susceptible to renal function compared with that of CT. We have previously reported that age-dependent significant increase in serum AcCT concentration was observed in healthy subjects,[40] although the level was not as high as that in CKD patients. Because eGFR declines with aging,[41] the increase of serum AcCT concentration in older healthy subjects may be associated with the decline of eGFR.

In addition, the results in cultured cells showed that both the mitochondrial and cytosolic concentrations of deuterium-labeled CT-d3 and AcCT-d3 derived from added CT-d3 were decreased by addition of AcCT-d0 in a dose-dependent manner. In HEK293 cells transfected with a human CT transporter, OCTN2, Ohashi *et al.*[42] showed that uptake of CT was inhibited by various types of acyl-CT, including AcCT, and there was no marked difference in Km values between AcCT (8.50 μM) and CT (4.34 μM). Thus, increased blood AcCT concentration appears to inhibit uptake of CT by skeletal muscles in CKD patients, which may also exacerbate insulin resistance.

Previous studies reported that insulin resistance in CKD patients improved after hemodialysis.[4,43] In addition, insulin resistance was induced by addition of sera from pre-hemodialyzed CKD patients to cultured adipocytes,[6] but not by addition of sera from post-hemodialyzed CKD patients.[7] AcCT (C2; MW = 204) is the shortest-chain acyl-CT and can pass through a dialysis membrane, similarly to carnitine (MW = 161),[44] while long-chain acyl-CTs are not dialyzed.[44] In our preliminary study, serum AcCT concentration (16.8 ± 2.5 μM) in CKD patients was significantly reduced to the healthy level after hemodialysis (6.3 ± 0.7 μM). Furthermore, we confirmed that effluent from hemodialysis contains abundant AcCT as well as CT (data not shown). These findings support our idea that elevated serum AcCT is a possible cause of insulin resistance in CKD patients. On the other hand, insulin resistance in metabolic syndrome is not likely to be caused by the elevated AcCT. We have preliminary observed that there was no significant elevation of serum AcCT concentration in non-alcoholic steatohepatitis (NASH) patients with insulin resistance (unpublished observation). Therefore, the

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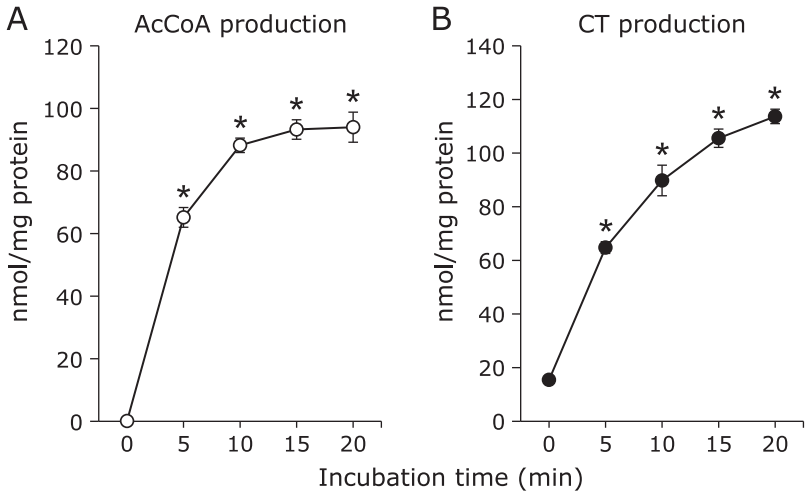

Fig. 6. AcCoA (A) and free CT (B) productions through CrAT activity in homogenized mitochondria exposed to AcCT and free CoA. Naïve mitochondria were isolated from C2C12 myotubes. Homogenized mitochondria were incubated with 500 μM AcCT and 500 μM free CoA at 37°C for up to 20 min. The production levels of AcCoA and free CT were calculated as per mitochondrial protein. Data are shown as mean ± 2 × SEM obtained in a quadruplicate assay. *p<0.001 (one-way ANOVA) vs the respective 0 min incubation.

increased serum AcCT concentration is caused mainly by the reduction of renal clearance and it is not a main cause of insulin resistance in metabolic syndrome without CKD.

In conclusion, serum AcCT concentrations in CKD patients were increased with reduction of renal function evaluated by eGFR. In muscle cell culture, exogenous AcCT was metabolized by mitochondrial CrAT and this resulted in elevation of the mitochondrial AcCoA/CoA ratio and insulin resistance. This mechanism partly explains insulin resistance in the patients with CKD.

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Abbreviations

AcCoA	acetyl-CoA
AcCT	acetylcarnitine
AcCT-d0	authentic acetyl-L-carnitine
AcCT-d3	acetyl-L-[$^2H_3$]carnitine
AcCT-d9	acetyl-DL-[$^2H_9$]carnitine
Acyl-CT	acylcarnitine
CACT	carnitine acylcarnitine translocase
CKD	chronic kidney disease
CoA	coenzyme A
CrAT	carnitine acetyltransferase
CT	carnitine
CT-d0	authentic L-carnitine
CT-d3	L-[$^2H_3$]carnitine
CT-d9	DL-[$^2H_9$]carnitine
2DG	2-deoxyglucose
eGFR	estimated glomerular filtration rate
ESI	electrospray ionization
FFA	free fatty acid
GLUT 4	glucose transporter 4
IR	insulin receptor
OCTN2	organic anion transporter type-2
PDH	pyruvate dehydrogenase
PDK	pyruvate dehydrogenase kinase
TCA	tricarboxylic acid

Conflict of Interest

No potential conflicts of interest were disclosed.

References

1 Horton ES, Johnson C, Lebovitz HE. Carbohydrate metabolism in uremia. *Ann Intern Med* 1968; **68**: 63–74.
2 Becker B, Kronenberg F, Kielstein JT, *et al*. Renal insulin resistance syndrome, adiponectin and cardiovascular events in patients with kidney disease: the mild and moderate kidney disease study. *J Am Soc Nephrol* 2005; **16**: 1091–1098.
3 Hosoya K, Minakuchi H, Wakino S, *et al*. Insulin resistance in chronic kidney disease is ameliorated by spironolactone in rats and humans. *Kidney Int* 2015; **87**: 749–760.
4 DeFronzo RA, Alvestrand A, Smith D, Hendler R, Hendler E, Wahren J. Insulin resistance in uremia. *J Clin Invest* 1981; **67**: 563–568.
5 Friedman JE, Dohm GL, Elton CW, *et al*. Muscle insulin resistance in uremic humans: glucose transport, glucose transporters, and insulin receptors. *Am J Physiol* 1991; **261 (1 Pt 1)**: E87–E94.
6 Maloff BL, McCaleb ML, Lockwood DH. Cellular basis of insulin resistance in chronic uremia. *Am J Physiol* 1983; **245**: E178–E184.
7 McCaleb ML, Izzo MS, Lockwood DH. Characterization and partial purification of a factor from uremic human serum that induces insulin resistance. *J Clin Invest* 1985; **75**: 391–396.
8 Bezaire V, Bruce CR, Heigenhauser GJ, *et al*. Identification of fatty acid translocase on human skeletal muscle mitochondrial membranes: essential role in fatty acid oxidation. *Am J Physiol Endocrinol Metab* 2006; **290**: E509–E515.
9 Bremer J. Carnitine--metabolism and functions. *Physiol Rev* 1983; **63**: 1420–1480.
10 Randle PJ, Garland PB, Hales CN, Newsholme EA. The glucose fatty-acid

cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet* 1963; **1**: 785–789.
11 Fritz IB, Schultz SK, Srere PA. Properties of partially purified carnitine acetyltransferase. *J Biol Chem* 1963; **238**: 2509–2517.
12 Stephens FB, Constantin-Teodosiu D, Greenhaff PL. New insights concerning the role of carnitine in the regulation of fuel metabolism in skeletal muscle. *J Physiol* 2007; **581**: 431–444.
13 Muoio DM, Noland RC, Kovalik JP, *et al*. Muscle-specific deletion of carnitine acetyltransferase compromises glucose tolerance and metabolic flexibility. *Cell Metab* 2012; **15**: 764–777.
14 Cao Y, Wang YX, Liu CJ, Wang LX, Han ZW, Wang CB. Comparison of pharmacokinetics of L-carnitine, acetyl-L-carnitine and propionyl-L-carnitine after single oral administration of L-carnitine in healthy volunteers. *Clin Invest Med* 2009; **32**: E13–E19.
15 Fouque D, Holt S, Guebre-Egziabher F, *et al*. Relationship between serum carnitine, acylcarnitines, and renal function in patients with chronic renal disease. *J Ren Nutr* 2006; **16**: 125–131.
16 Stanley CA. Carnitine deficiency disorders in children. *Ann N Y Acad Sci* 2004; **1033**: 42–51.
17 Maeda Y, Ito T, Suzuki A, *et al*. Simultaneous quantification of acylcarnitine isomers containing dicarboxylic acylcarnitines in human serum and urine by high-performance liquid chromatography/electrospray ionization tandem mass spectrometry. *Rapid Commun Mass Spectrom* 2007; **21**: 799–806.
18 Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group. KDIGO 2012 clinical practice guideline for the evaluation and management of chronic kidney disease. *Kidney Int Suppl* 2013; **3**: 1–150.
19 Imai E, Horio M, Nitta K, *et al*. Estimation of glomerular filtration rate by the MDRD study equation modified for Japanese patients with chronic kidney disease. *Clin Exp Nephrol* 2007; **11**: 41–50.
20 Ghoshal AK, Guo T, Soukhova N, Soldin SJ. Rapid measurement of plasma acylcarnitines by liquid chromatography-tandem mass spectrometry without derivatization. *Clin Chim Acta* 2005; **358**: 104–112.
21 Miyazaki T, Honda A, Ikegami T, *et al*. Simultaneous quantification of salivary 3-hydroxybutyrate, 3-hydroxyisobutyrate, 3-hydroxy-3-methylbutyrate, and 2-hydroxybutyrate as possible markers of amino acid and fatty acid catabolic pathways by LC-ESI-MS/MS. *Springerplus* 2015; **4**: 494.
22 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; **193**: 265–275.
23 Honda A, Salen G, Matsuzaki Y, *et al*. Differences in hepatic levels of intermediates in bile acid biosynthesis between Cyp27(–/–) mice and CTX. *J Lipid Res* 2001; **42**: 291–300.
24 Blachnio-Zabielska AU, Koutsari C, Jensen MD. Measuring long-chain acyl-coenzyme A concentrations and enrichment using liquid chromatography/tandem mass spectrometry with selected reaction monitoring. *Rapid Commun Mass Spectrom* 2011; **25**: 2223–2230.
25 Seiler SE, Martin OJ, Noland RC, *et al*. Obesity and lipid stress inhibit carnitine acetyltransferase activity. *J Lipid Res* 2014; **55**: 635–644.
26 Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Methods* 1986; **89**: 271–277.
27 Noland RC, Koves TR, Seiler SE, *et al*. Carnitine insufficiency caused by aging and overnutrition compromises mitochondrial performance and metabolic control. *J Biol Chem* 2009; **284**: 22840–22852.
28 Idell-Wenger JA, Grotyohann LW, Neely JR. Coenzyme A and carnitine distribution in normal and ischemic hearts. *J Biol Chem* 1978; **253**: 4310–4318.
29 Linn TC, Pettit FH, Reed LJ. Alpha-keto acid dehydrogenase complexes. X. Regulation of the activity of the pyruvate dehydrogenase complex from beef kidney mitochondria by phosphorylation and dephosphorylation. *Proc Natl Acad Sci U S A* 1969; **62**: 234–241.
30 Yeaman SJ. The 2-oxo acid dehydrogenase complexes: recent advances. *Biochem J* 1989; **257**: 625–632.
31 Kerbey AL, Randle PJ, Cooper RH, Whitehouse S, Pask HT, Denton RM. Regulation of pyruvate dehydrogenase in rat heart. Mechanism of regulation of proportions of dephosphorylated and phosphorylated enzyme by oxidation of fatty acids and ketone bodies and of effects of diabetes: role of coenzyme A, acetyl-coenzyme A and reduced and oxidized nicotinamide-adenine dinucleotide. *Biochem J* 1976; **154**: 327–348.
32 Sugden MC, Holness MJ. Recent advances in mechanisms regulating glucose oxidation at the level of the pyruvate dehydrogenase complex by PDKs. *Am J Physiol Endocrinol Metab* 2003; **284**: E855–E862.
33 Wu P, Sato J, Zhao Y, Jaskiewicz J, Popov KM, Harris RA. Starvation and diabetes increase the amount of pyruvate dehydrogenase kinase isoenzyme 4 in rat heart. *Biochem J* 1998; **329 (Pt 1)**: 197–201.
34 Hoy AJ, Brandon AE, Turner N, *et al*. Lipid and insulin infusion-induced skeletal muscle insulin resistance is likely due to metabolic feedback and not changes in IRS-1, Akt, or AS160 phosphorylation. *Am J Physiol Endocrinol Metab* 2009; **297**: E67–E75.
35 Fueger PT, Lee-Young RS, Shearer J, *et al*. Phosphorylation barriers to skeletal and cardiac muscle glucose uptakes in high-fat fed mice: studies in mice with a 50% reduction of hexokinase II. *Diabetes* 2007; **56**: 2476–2484.
36 Furler SM, Jenkins AB, Storlien LH, Kraegen EW. *In vivo* location of the rate-limiting step of hexose uptake in muscle and brain tissue of rats. *Am J Physiol* 1991; **261 (3 Pt 1)**: E337–E347.
37 Furler SM, Oakes ND, Watkinson AL, Kraegen EW. A high-fat diet influences insulin-stimulated posttransport muscle glucose metabolism in rats. *Metabolism* 1997; **46**: 1101–1106.
38 Katz A, Raz I, Spencer MK, Rising R, Mott DM. Hyperglycemia induces accumulation of glucose in human skeletal muscle. *Am J Physiol* 1991; **260 (4 Pt 2)**: R698–R703.
39 Guarnieri G, Situlin R, Biolo G. Carnitine metabolism in uremia. *Am J Kidney Dis* 2001; **38 (4 Suppl 1)**: S63–S67.
40 Iwamoto J, Honda A, Miyamoto Y, *et al*. Serum carnitine as an independent biomarker of malnutrition in patients with impaired oral intake. *J Clin Biochem Nutr* 2014; **55**: 221–227.
41 Musso CG, Oreopoulos DG. Aging and physiological changes of the kidneys including changes in glomerular filtration rate. *Nephron Physiol* 2011; **119 Suppl 1**: p1–p5.
42 Ohashi R, Tamai I, Yabuuchi H, *et al*. Na(+)-dependent carnitine transport by organic cation transporter (OCTN2): its pharmacological and toxicological relevance. *J Pharmacol Exp Ther* 1999; **291**: 778–784.
43 DeFronzo RA, Smith D, Alvestrand A. Insulin action in uremia. *Kidney Int Suppl* 1983; **16**: S102–S114.
44 Evans AM, Faull R, Fornasini G, *et al*. Pharmacokinetics of L-carnitine in patients with end-stage renal disease undergoing long-term hemodialysis. *Clin Pharmacol Ther* 2000; **68**: 238–249.

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