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RESEARCH ARTICLE

Multiple AMPK activators inhibit L-carnitine uptake in C2C12 skeletal muscle myotubes

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Shaw A, Jeromson S, Watterson KR, Pediani JD, Gallagher IJ, Whalley T, Dreczkowski G, Brooks N, Galloway SD, Hamilton DL. Multiple AMPK activators inhibit L-carnitine uptake in C2C12 skeletal muscle myotubes. *Am J Physiol Cell Physiol* 312: C689–C696, 2017. First published March 15, 2017; doi:10.1152/ajpcell.00026.2016.—Mutations in the gene that encodes the principal L-carnitine transporter, OCTN2, can lead to a reduced intracellular L-carnitine pool and the disease Primary Carnitine Deficiency. L-Carnitine supplementation is used therapeutically to increase intracellular L-carnitine. As AMPK and insulin regulate fat metabolism and substrate uptake, we hypothesized that AMPK-activating compounds and insulin would increase L-carnitine uptake in C2C12 myotubes. The cells express all three OCTN transporters at the mRNA level, and immunohistochemistry confirmed expression at the protein level. Contrary to our hypothesis, despite significant activation of PKB and 2DG uptake, insulin did not increase L-carnitine uptake at 100 nM. However, L-carnitine uptake was modestly increased at a dose of 150 nM insulin. A range of AMPK activators that increase intracellular calcium content [caffeine (10 mM, 5 mM, 1 mM, 0.5 mM), A23187 (10 μM)], inhibit mitochondrial function [sodium azide (75 μM), rotenone (1 μM), berberine (100 μM), DNP (500 μM)], or directly activate AMPK [AICAR (250 μM)] were assessed for their ability to regulate L-carnitine uptake. All compounds tested significantly inhibited L-carnitine uptake. Inhibition by caffeine was not dantrolene (10 μM) sensitive despite dantrolene inhibiting caffeine-mediated calcium release. Saturation curve analysis suggested that caffeine did not competitively inhibit L-carnitine transport. To assess the potential role of AMPK in this process, we assessed the ability of the AMPK inhibitor Compound C (10 μM) to rescue the effect of caffeine. Compound C offered a partial rescue of L-carnitine uptake with 0.5 mM caffeine, suggesting that AMPK may play a role in the inhibitory effects of caffeine. However, caffeine likely inhibits L-carnitine uptake by alternative mechanisms independently of calcium release. PKA activation or direct interference with transporter function may play a role.

carnitine uptake; AMPK; insulin; kinase assays

L-CARNITINE [3-hydroxy-4-(trimethylazaniumyl) butanoate] is a dipeptide compound which acts as a cofactor for the transport of long chain fatty acids into the mitochondria where they can be oxidized (12). It is synthesized from methionine and lysine primarily in the liver but also in the brain and kidneys. Ninety-five percent of carnitine in the body is stored within skeletal muscle (6). Carnitine transport into skeletal muscle cells occurs largely via sodium-dependent symport through the organic cation transporter novel type 2 (OCTN2) (40). Primary Carnitine Deficiency is associated with mutations in the SLC22A4 gene, which codes for OCTN2 and results in a reduced intramuscular L-carnitine pool (10, 29). Manifestations of this disorder are either cardiac, skeletal muscle, or metabolically related. The most common diseases associated with each manifestation are dilated cardiac myopathy, hypotonia, and hypoglycemia, respectively (10, 45). Low L-carnitine content is particularly damaging to the heart and leads to a compromised ability to utilize fatty acids for ATP synthesis and can ultimately lead to heart failure (45). The threshold for the intramuscular pool of L-carnitine at which these manifestations occur is yet to be defined.

L-Carnitine supplementation has also been implicated as a way to manipulate carbohydrate and fat metabolism to improve either exercise performance or metabolic function (48). Indeed there are studies supporting this in a number of tissues and contexts. For instance: increased L-carnitine delivery can have an insulin-mimetic effect on ex vivo tissue (42); dual L-carnitine and insulin infusion alters skeletal muscle fuel selection (49), whereas oral L-carnitine supplementation alters whole body glucose handling under an OGTT (13). Furthermore, Stephens et al. (52) attributed their finding of reduced adiposity in subjects fed a caloric surplus plus L-carnitine to greater fat oxidation during low-intensity exercise due to greater capacity for fatty acid transport and oxidation. L-Carnitine supplementation studies have demonstrated a number of equivocal findings in terms of exercise performance (46). This is largely believed to be due to the difficulty in altering the intramuscular L-carnitine pool. However, in two papers from the same study (52, 57), it appears that muscle L-carnitine content can be elevated when supplemented with high doses of carbohydrate (80 g twice per day), indicating that perhaps insulin may play a role in regulating L-carnitine uptake and or accumulation. Currently there is a significant gap in the literature concerning the molecular and pharmacological regulation of L-carnitine transport into skeletal muscle.

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As we mentioned, insulin has been proposed to play a role in L-carnitine transport (or at the very least accumulation) (52, 57). However, another key regulator of substrate transport, which has to our knowledge never been considered as a potential regulator of L-carnitine transport, is AMP-activated protein kinase (AMPK). Some have speculated that L-carnitine levels may regulate AMPK function (14), but this has never been experimentally confirmed. AMPK is a cellular energy sensor activated by a decrease in the ATP-to-AMP ratio (18). A decrease in the ATP-to-AMP ratio leads to the activation of AMPK during periods of energy stress such as muscle contraction or metabolic toxicity (18). AMPK acts to restore the ATP-to-AMP ratio by inhibiting energy-consuming pathways (such as protein and fatty acid synthesis) and activating energy-generating pathways (such as glucose uptake and fatty acid oxidation) (9). The pharmacological activation of AMPK via the AMP mimetic AICAR has previously been shown to drive substrate uptake including glucose and fatty acids (3, 4, 43, 47). Similar to AMPK, insulin is well characterized as a driver for glucose (1), amino acid (22), and fatty acid uptake (32). If AMPK were to drive L-carnitine uptake then strategies similar to the co-ingestion of L-carnitine with carbohydrate (52, 57) could be developed to enhance skeletal muscle L-carnitine content.

We hypothesized that insulin and a range of AMPK-activating compounds would enhance the transport of L-carnitine into C2C12 skeletal muscle myotubes. Contrary to our hypothesis, however, we find that insulin had only a modest effect on L-carnitine uptake at 150 nM, and all AMPK-activating reagents inhibited L-carnitine uptake. These data have wider implications for the pharmacological treatment of L-carnitine deficiency disorders and optimizing L-carnitine accumulation in skeletal muscle to enhance metabolism.[1]

METHODS

Materials. PKB α/β- and γ-specific antibodies were sourced from the DSTT (Dundee University), and (AMPK) α1- and α2-specific antibodies were produced by GL Biochem (Shanghai, China) against the following antigens; α1, CTSPPDSFLDDHHLTR and α2, CMDDSAMHIPPGLKPH. The OCTN1/2/3 antibody was sourced from Santa Cruz Biotechnology. Radioactive tracers were provided by Hartmann Analytic. All other reagents and laboratory consumables were sourced from Fisher Scientific unless otherwise stated.

Cell culture. C2C12 myoblasts (ATCC) were cultured in 6- or 12-well plates and maintained in a growth media containing high glucose Dulbecco's modified Eagle's medium (DMEM), 20% fetal bovine serum (FBS), and a 1% penicillin-streptomycin. Once 80–100% confluent, the cells were differentiated using a differentiating media containing high glucose DMEM, 2% donor horse serum, and 1% penicillin-streptomycin. Experiments were carried out once cells were fully differentiated into myotubes (3–5 days after differentiation).

Substrate transport assays. Cells were placed in a serum-free media for 3 h before the experiment. After being serum starved, the cells were exposed to a tritiated L-carnitine buffer [100 μM (or 0.1 μM for insulin stimulation) at 80 μCi/μMol] and each treatment was completed with the addition of insulin (100 nM and 150 nM) or one of the following AMPK activators: AICAR [250 μM (previously shown to activate AMPK at 500 μM) (23)], 2,4-dinitrophenol (DNP) [500 μM (activates AMPK in L6 myotubes at 500 μM) (39)], rotenone [1 μM (previously demonstrated to activate AMPK in the low micromolar range) (20)], berberine [100 μM (30)], caffeine [5 mM (37)], A23187 [10 μM (19)], and sodium azide [75 μM (7)]. Following 3 h of incubation, the reaction was stopped using ice-cold 0.9% saline. The cells were lysed using sodium hydroxide (NaOH) lysis buffer and collected in Gold Star LT Quanta scintillation fluid (Meridian Biotechnologies, Chesterfield, UK) for scintillation counting (United Technologies Packard 2200CA TriCarb). Uptake data were normalized back to protein content as assessed by Bradford assays.

For glucose uptake, cells were serum starved in serum and amino acid-free PBS + 5 mM glucose for 3 h. After the 3 h, serum-starved cells were stimulated with or without 100 nM insulin for 30 min. Following the 30 min, insulin stimulation glucose uptake assays were carried out using a buffer containing tritiated 2-deoxyglucose (10 μM at 0.66 μCi/ml) for 10 min at room temperature. Ice-cold saline (0.9% NaCl) was used to stop the reaction, and cells were lysed using sodium hydroxide (NAOH) lysis buffer and added to Gold Star LT Quanta scintillation fluid (Meridian Biotechnologies) for scintillation counting (United Technologies Packard 2200CA TriCarb). Uptake data were normalized back to protein content as assessed by Bradford assays.

Biochemical assays. All kinase assays were carried out by immunoprecipitation (IP) for 2 h at 4°C as previously described (35). For immunohistochemistry, cells were fixed using 4% formaldehyde in PBS and stored at 4°C. Slides were blocked in 5% BSA, washed in phosphate-buffered saline (PBS), and exposed overnight at 4°C to rabbit OCTN1/2/3 polyclonal antibodies (Santa Cruz Biotechnology). Sections were washed in PBS, incubated in the dark at room temperature for 1 h with an Alexa 555-conjugated goat anti-rabbit immunoglobulin G (IgG; 1:1,000; Abcam), and mounted with fluorescent mounting medium (DAKO) containing DAPI. Slides were stored in the dark at 20°C until fluorochromes were activated by use of a fluorescent microscope.

For the calcium imaging experiments, C2C12 myoblasts were plated onto glass coverslips and then differentiated to myotubes as described previously. Differentiated cells were then loaded with 3 μM calcium-sensitive dye, Fura-2 (Sigma Aldrich, Dorset, UK) in Locke's buffer medium [154 mM NaCl, 4 mM $NaHCO_3$, 5 mM KCl, 2.3 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, 10 mM HEPES (pH 7.4)] in the presence of 0.025% pluronic F-127 (Life Technologies, Paisley, UK) at 37°C for 45 min. The cells were then washed ×3 in Locke's buffer, and the coverslips were placed into a microscope chamber containing Locke's buffer. The cells were then illuminated with an ultra high point intensity 75-W xenon arc lamp (Optosource, Cairn Research, Faversham, Kent, UK) and imaged using a Nikon Diaphot inverted microscope equipped with a Nikon ×40 oil immersion Fluor objective lens (numerical aperture = 1.3) and a monochromator (Optoscan, Cairn Research), which was used to alternate the excitation wavelength between 340 nm and 380 nm. Fura-2 fluorescence emission at 510 nm was monitored using a high-resolution interline-transfer cooled digital CCD camera (Cool Snap-HQ, Roper Scientific/Photometrics, Tucson, AZ). MetaFluor imaging software (Universal Imaging, Downing, PA) was used for the control of the monochromator, CCD camera, and for processing of the cell image data. Caffeine (5 mM) was added after 60 s using a perfusion system. To measure the effect of dantrolene, the cells were preincubated for 20 min with 10 μM dantrolene [demonstrated to be effective at inhibiting calcium release in L6 myotubes (37)] before perfusion of 5 mM caffeine. Ratiometric images captured at 2-s intervals were then analyzed using MetaFluor software. Changes in calcium are expressed as fold changes over normalized basal calcium for each cell. Data were collected for 23–33 cells over three separate experiments, and statistical differences between mean maximal caffeine-induced calcium responses in the presence and absence of dantrolene were analyzed

[1] This article is the topic of an Editorial Focus by Da Xu and Guofeng You (59).

using a two-tailed unpaired *t*-test with Excel software, with significance determined as $P < 0.05$.

Primers for PCR analysis were designed from reference sequences using Primer 3 (Table 1). RNA was extracted from C2C12 cells using TRIzol reagent (Invitrogen) following standard protocols. One microgram total RNA was reverse transcribed using oligo(dT) primers. Buffer, reverse transcriptase, dNTPs, and RNAse inhibitor were combined as directed by the manufacturer (RevertAid RT kit; Fisher), and 8 μl were added to each RNA/oligo(dT) cocktail. Reactions were incubated for 60 min at 42°C with a final termination step of 70°C for 5 min. cDNA was stored at −20°C before use. Amplification of gene products was carried out using the Fermentas PCR kit (Fermentas, Thermo-Scientific). Briefly, 1 μl of cDNA was combined with 12.5 μl supplied PCR master mix, 1 μl of forward and reverse primer each at 10 μM, and 9.5 μl ddH_2O. The reaction conditions were 95°C for 1 min, 35 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 60 s followed by a final 5 min at 72°C. Products were visualized by agarose gel electrophoresis in 1% agarose gel. Electrophoresis was carried out for 40 min at 100 V. Bands were imaged with a Bio-Rad gel doc system (Bio-Rad).

Statistics. Unless otherwise stated, figures were created and statistical analyses were carried out using the GraphPad Prism 4 platform. Paired *t*-tests or repeated-measures ANOVA with a post hoc Tukey's HSD test were used to determine significance between control and treatment groups.

RESULTS

C2C12 myotubes express OCTN1/2/3. Previous work has shown that primary human myotubes and C2C12 myoblasts and myotubes transport L-carnitine (15, 34). However, to ensure that our C2C12 model would be a useful tool to study L-carnitine transport, we first assessed whether the OCTN family of transporters that shuttle L-carnitine into the cell were present in the C2C12 cell line. We carried out PCR reactions to test for the presence of the OCTN1/2/3 transcript isoforms with isoform-specific primers. PCR products at the predicted weights were found for all three sets of primers, indicating the presence of OCTN1/2/3 mRNA (Fig. 1*A*). To confirm expression at the protein level, we carried out immunohistochemistry experiments with a pan-OCTN1/2/3 antibody and identified that C2C12 myotubes expressed some or all of these isoforms at the protein level. Staining can be seen throughout the identified myotube membrane, indicating the presence of the transporter. (Fig. 1*B*).

L-Carnitine uptake and insulin. Insulin stimulates the uptake of glucose (1) and small neutral amino acids by activating the systemA transport system (22). We hypothesized that insulin may also regulate the transport of L-carnitine. We first confirmed that our cells were insulin responsive by demonstrating insulin-stimulated PKB activation and glucose uptake (Fig. 2, *A* and *B*). Despite the obvious insulin responsiveness of our cell line, we found that 100 nM did not have a significant effect on L-carnitine uptake; however, 150 nM insulin did induce a modest but significant increase in uptake (Fig. 2*C*).

Table 1. *Primer details for Mus musculus Octn amplification*

Primer	Sequence
MmOctn1_F	GGTGGAAACATGCGGGACTA
MmOctn1_R	GATGATGCGAACCGACTTGC
Mm_Octn2_F	CCACGGTGTCCCCTTATTCC
Mm_Octn2_R	TTGCGACCAAACCTGTCTGA
Mm_Octn3_F	CGACGCTTTCTCGAACTCCT
Mm_Octn3_R	CACCATGAAGCCAAACGCAA

F, forward; R, reverse.

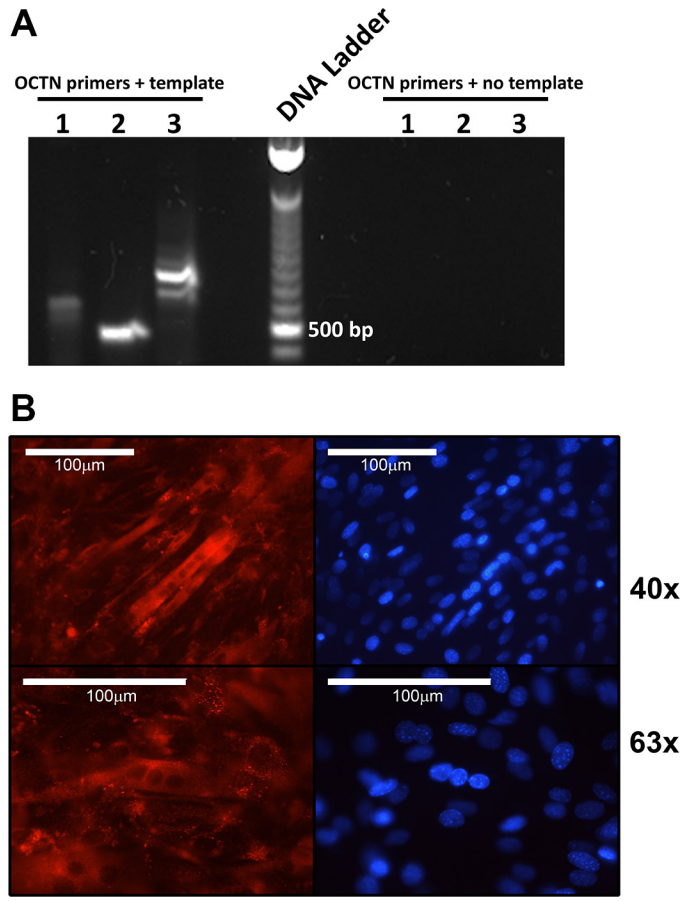

Fig. 1. Expression of carnitine transporter isoforms in C2C12 myotubes. PCR amplification demonstrates the expression of the three OCTN isoforms in C2C12 myotubes (*A*). Immunohistochemical analysis with a pan OCTN1/2/3 antibody demonstrates expression at the protein level (*B*). Blue indicates DAPI staining of the nucleus and red indicates OCTN1/2/3.

AMPK activation by a range of confirmed AMPK activators. From previous studies we know that AMPK, like insulin, drives substrate uptake (3–5, 47). With this in mind, we aimed to evaluate the potential role of AMPK in L-carnitine uptake. First, however, we identified seven "AMPK-activating compounds" and determined their ability to induce AMPK activity in C2C12 myotubes. All compounds tested significantly activated AMPK after a 30-min stimulation period to varying degrees, ranging from 2.2 ± 0.58-fold to 5.42 ± 0.48-fold (Fig. 3*B*). Having confirmed that each compound significantly activates AMPK we next evaluated the effect of these compounds on L-carnitine uptake (Fig. 3*A*). Contrary to our hypothesis, treatment of C2C12 myotubes with these compounds results in a significant reduction in L-carnitine uptake in all treatment conditions to varying degrees, from 32.61 ± 3.05% reduction to 81.16 ± 5.85% reduction.

Exploring the mechanism of caffeine mediated L-carnitine uptake. There was a linear relationship between the degree of activation of AMPK and the degree of inhibition of L-carnitine uptake (data not shown); however, caffeine was a substantial outlier on this curve and so we further explored the mechanism of action of caffeine on L-carnitine uptake. Although caffeine is

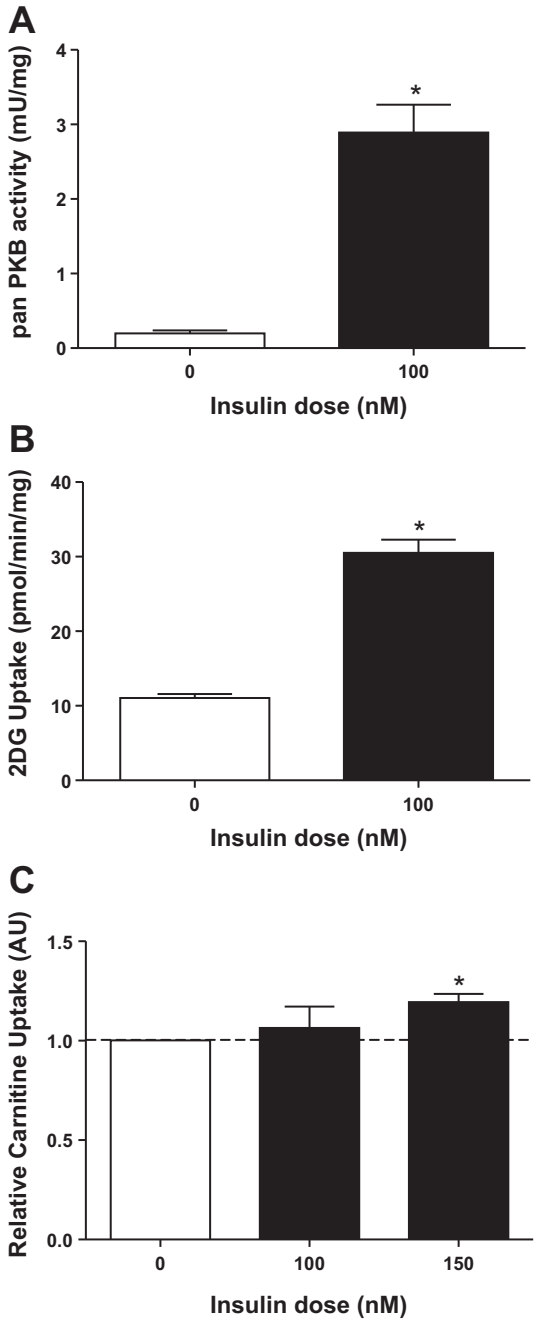

Fig. 2. Insulin-mediated carnitine transport. C2C12 myotubes were serum starved in PBS containing 5 mM glucose for 2 h followed by a 30-min stimulation with insulin to assess PKB activity (*A*), 2DG uptake (*B*), and L-carnitine transport ($n = 4$). *Significantly different from baseline ($P < 0.05$).

not an organic cation, we performed a substrate-saturation curve to assess whether caffeine could be inhibiting uptake via competitive inhibition of transport; as expected, the kinetic curve revealed that the predicted V_{max} was reduced indicating, noncompetitive inhibition (Fig. 4*A*). Further analysis of inhibition across a range of substrate concentrations revealed a dose-dependent effect of caffeine across all substrate concentrations tested (Fig. 4*B*). To test the calcium dependence of the effects of caffeine, we assessed the impact of 10 μM dantrolene on the reversibility of the inhibition effect of caffeine. As expected, dantrolene had no impact on the inhibitory effect of A23187 (a calcium ionophore); however, neither did it reverse the inhibitory effects of caffeine (Fig. 4*C*). To test whether a lower dose of caffeine could be reversed by dantrolene, we halved the dose of caffeine to 5 mM and again dantrolene did not rescue the uptake inhibition (Fig. 4*D*). This is despite our findings that dantrolene substantially inhibited the appearance of calcium in response to caffeine stimulation (Fig. 4*E*). We next assessed whether the inhibitory effect was dependent on AMPK by using the inhibitor Compound C. These data revealed a partial rescue of carnitine transport at a relatively low dose of caffeine (0.5 mM) but not at the higher dose of caffeine (5 mM), suggesting potential alternative mechanisms in the control of L-carnitine transport.

DISCUSSION

The transport of L-carnitine into skeletal muscle is an essential process for optimal metabolic functioning. The inability to transport L-carnitine efficiently is associated with Primary Carnitine Deficiency, muscle weakness, and, in C2C12 myotubes, impaired growth (15). L-Carnitine transport into skeletal muscle cell lines has two transport affinities, one at a high affinity within the physiological range for L-carnitine and a low-affinity transport activity that works at higher, nonphysiological ranges (15, 34). Here we investigated the pharmacological regulation of L-carnitine uptake in C2C12 myotubes across these affinity ranges for L-carnitine transport during

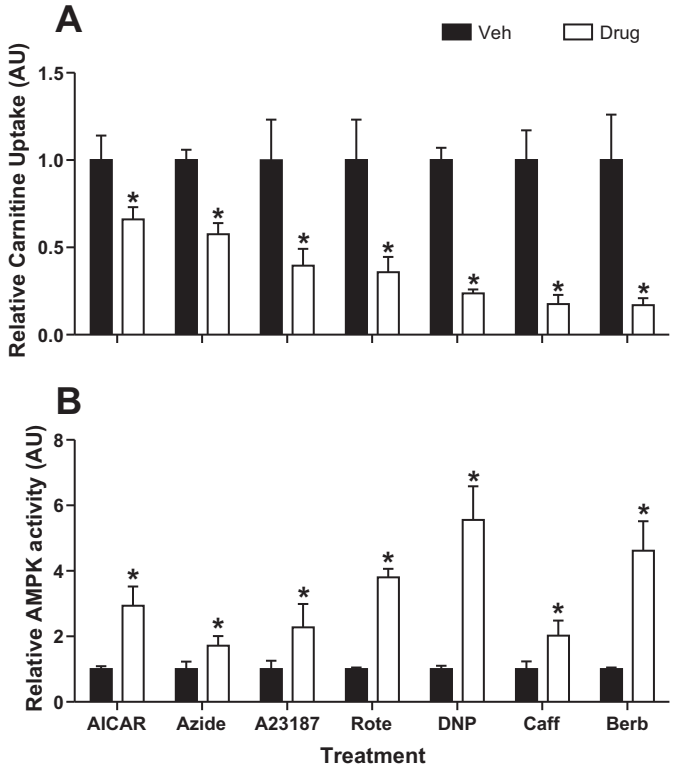

Fig. 3. AMPK activators inhibit L-carnitine transport. Cells were treated with AICAR (250 μM), azide (75 μM), berberine (100 μM), DNP (500 μM), caffeine (10 mM), and A23187 (10 μM). L-Carnitine transport was determined (*A*) and AMPK activity was assessed (*B*). Compounds were present throughout the uptake assay and data were normalized to each respective control. *Significantly different from baseline.

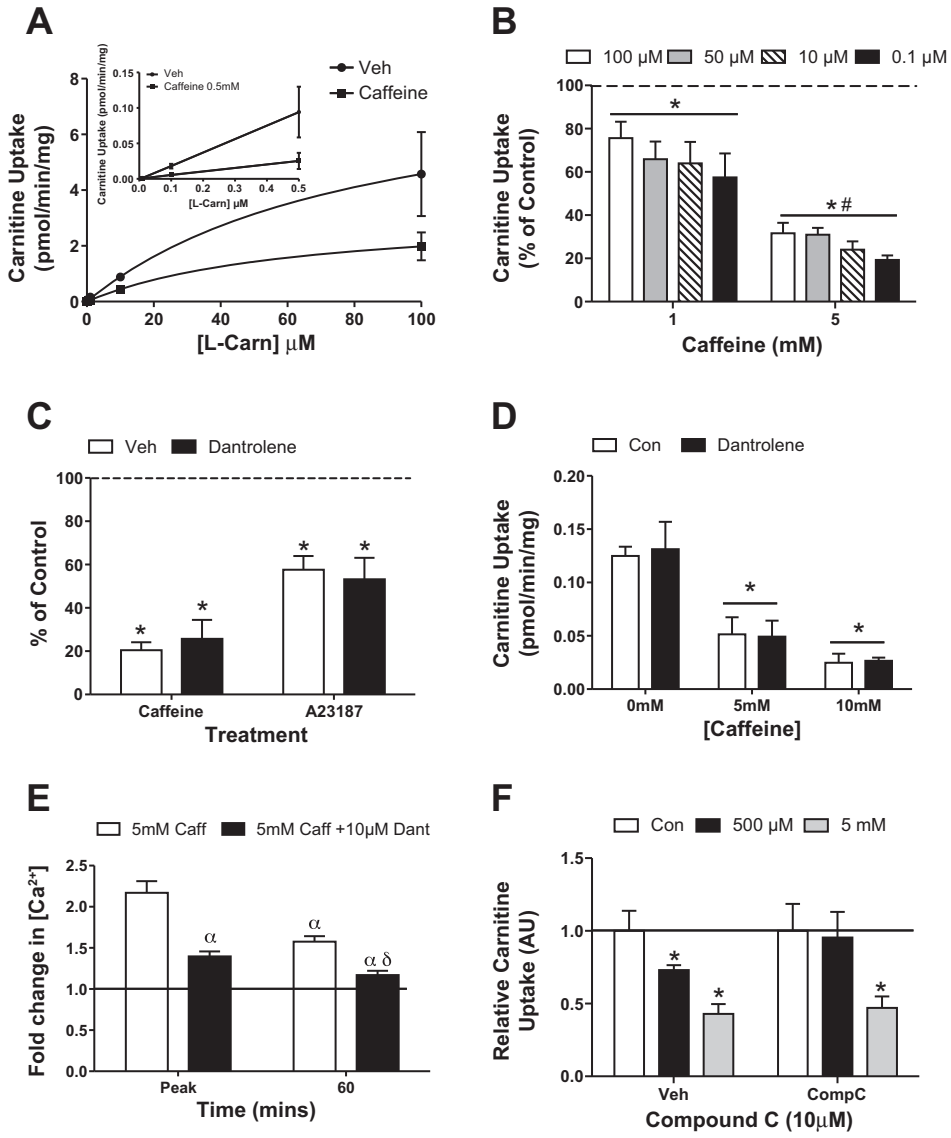

Fig. 4. Caffeine inhibits L-carnitine transport independently of calcium release. Uptake kinetics were assessed with increasing amounts of tracee in the presence or absence of 5 mM caffeine (*A*). Inhibition by caffeine was assessed with 1 or 5 mM caffeine at 100, 50, 10, or 0.1 μM L-carnitine (*B*). Cells were pretreated with dantrolene (10 μM) before the addition of caffeine during the uptake and calcium imaging experiments. Caffeine (10 mM) or A23187 (10 μM) was added following 10 min of preincubation with dantrolene (10 μM), and L-carnitine uptake was assessed. Data are represented as % of baseline (*C*). L-Carnitine uptake was assessed with 0 mM, 5 mM, and 10 mM caffeine with or without 10 μM dantrolene (*D*). Calcium release was assessed as described in METHODS with 5 mM caffeine with or without 10 μM dantrolene (*E*). Uptake of L-carnitine was assessed with 0.5 mM or 5 mM caffeine in the presence or absence of 10 μM Compound C (*F*). *Significantly different from baseline. #Significantly different from 1 mM caffeine. αSignificantly different from 5 mM caffeine at peak. δSignificantly different from 5 mM caffeine at 60 min.

pharmacologically induced energy stress and in response to insulin. For the first time, we demonstrate that both direct and indirect AMPK activators inhibit the uptake of L-carnitine into the cytosol of C2C12 myotubes in a manner independent of calcium release and partially sensitive to the AMPK inhibitor Compound C. In addition, we provide evidence that insulin regulates L-carnitine uptake in this skeletal muscle model at the L-carnitine concentrations we used.

It is well documented that AMPK and insulin drive glucose and fatty acid uptake in striated muscle (3–5, 32, 47). Additionally, insulin directly regulates the uptake of small neutral amino acids (22). Given the role of L-carnitine in fatty acid metabolism (48), and the role that AMPK plays in promoting whole body fatty acid flux (21), we hypothesized that both insulin and AMPK would stimulate L-carnitine uptake. However, we found that an inverse relationship existed between AMPK activation and L-carnitine uptake and this raises the question as to why AMPK activators and potentially AMPK inhibit L-carnitine uptake. L-Carnitine uptake in skeletal muscle is ouabain sensitive and dependent on extracellular sodium (15). Therefore, Na^+-K^+-ATPase is required for skeletal muscle L-carnitine uptake; it is therefore possible that AMPK activation or energy stress may influence the Na^+ gradient required for transport (15). However, energy stress and specifically AMPK activation in skeletal muscle enhances the activity of the Na^+-K^+-ATPase (2). As a result it is unlikely that the compounds would have been working through the inhibition of the Na^+ gradient. It has been speculated, however, that the insulin-dependent activation of the Na^+-K^+-ATPase may be responsible for the increased L-carnitine accumulation/retention noted in human studies where L-carnitine is fed in conjunction with large doses of carbohydrate (51, 57) or infused with physiologically high insulin concentrations (50). However, none of these studies directly assessed L-carnitine transport. One study, however, demonstrated improved L-carnitine balance across the forearm following the consumption of 80 g of carbohydrate (44). The present study has shown that, at the most basic level, in cell culture, insulin does not affect the transport of L-carnitine in skeletal muscle unless a relatively high dose of insulin (150 nM vs. 100 nM) is utilized, whereas,

in humans, insulin has not been shown to affect L-carnitine uptake but rather balance and accumulation. Our data suggest that perhaps if insulin achieves a high enough concentration it could modify skeletal muscle uptake.

Analogs of L-carnitine including mildronate have the capacity to inhibit transport in skeletal muscle (15). Therefore, some of the compounds may inhibit L-carnitine uptake by competitive inhibition through shared transport mechanisms. For instance, berberine has been shown to not only be a potent AMPK activator (8, 25, 28, 31, 33) but also to be a substrate for and inhibitor of the subfamily of OCTN-related transporters OCT2/3, with an IC_{50} of 0.1–10 μM in MDCK cells transfected with hOCT2/3 (54). Metformin is also transported via a similar mechanism (38), and we avoided the use of this compound for that reason. Therefore the inhibition of L-carnitine uptake with 100 μM berberine could be due to competitive inhibition. Analysis of overexpressed OCT transcripts in transport models has revealed that OCT transporters transport a wide range of structurally diverse compounds (24). Some evidence suggests that a positive charge is also not obligatory for transport (26). Typically, however, they have been reported to transport small (60–350 Da) water-soluble compounds, usually containing a positively charged amine at physiological pH (24). The transport function of the OCTN subfamily, however, is far less characterized, but OCTN2 has undergone extensive testing for compounds that could interfere with transport function (56). These data suggest that a permanent positive charge is important but not entirely necessary to inhibit OCTN2 function (56). The metabolic toxins used in this study (DNP, rotenone, azide) have never been assessed as substrates of OCTN transporters, as far as we know. Additionally, they are not cations. Therefore, the mechanism of inhibition by these compounds is unlikely to be via competitive inhibition of transport, but it cannot be ruled out entirely. One potential mechanism could be due to the effect that mitochondrial toxins would have on enhancing glycolysis. The increased dependence on glycolysis and anaerobic respiration would lead to an acidification of the cellular environment which could cause an inhibition of L-carnitine transport which requires an optimal pH range to function fully (15). One interesting aspect, however, is that OCTN1 has been identified as having a nucleotide-binding site and displays transport function inhibited by ATP depletion by glycolytic inhibitors and mitochondrial toxins (55). These data suggest that transport of L-carnitine by OCTN1, at least, could be inhibited directly by reductions in ATP. This could also be a potential mechanism by which the AMP mimetic AICAR could work. If ZMP instead of ATP occupies the nucleotide-binding site it may impair function. However, it must be stated that the nucleotide dependency of OCTN transporters has never been confirmed nor has the ability of AMP to regulate function.

All of the compounds mentioned above must be transported into the cell to act on various intracellular molecules or directly on the mitochondria. Owing to the broad range of molecules that could act as substrates (56) for the OCTN transport mechanisms that transport L-carnitine, it is possible that the above compounds could interfere as competitive inhibitors of transport. The calcium release compounds, however, act either by increasing the permeability of the plasma membrane to Ca^{2+} (41) or by inducing Ca^{2+} release from the sarcoplasmic reticulum (53). Both are lipophilic molecules capable of diffusing across the membrane independently of active transport mechanisms (11, 41) and therefore competitive inhibition is unlikely to be the mode of action. Both molecules significantly activated AMPK by ~2-fold and both molecules inhibited L-carnitine uptake by ~50% (A23187) and ~80% (caffeine). Dantrolene (10 μM), which inhibits the release of calcium from intracellular stores (27), significantly inhibited calcium release with 5 mM caffeine; surprisingly, it did not rescue the inhibitory effect of caffeine on L-carnitine uptake. These data suggested that the influence of caffeine, at least, was independent of changes in Ca^{2+}. We therefore assessed the impact of caffeine on L-carnitine transport kinetics, which confirmed that caffeine was noncompetitively inhibiting transport. We next tested the dependency of the inhibitory effects of caffeine on AMPK with the AMPK inhibitor Compound C (10 μM). Compound C did not rescue the effect of caffeine at high doses (5 mM) but did partially recover uptake at a much lower dose of caffeine (0.5 mM), suggesting that the inhibition of uptake was at least partially dependent on AMPK.

It is clear from these data that alternative caffeine-sensitive mechanisms exist in the inhibition of L-carnitine transport. Caffeine also inhibits phosphodiesterases influencing cAMP levels (36). cAMP activates PKA and the effects of caffeine on *Drosophila* brain have been shown to be sensitive to PKA inhibitors (58). Therefore in addition to AMPK, there could be a role for PKA in the effects of caffeine on L-carnitine transport and it would therefore be interesting to assess the influence of forskolin, a PKA activator, on L-carnitine transport. Alternatively, caffeine could be interfering with transport function through an as yet unidentified mechanism. Regardless of the mechanism, our data showed that caffeine was inhibitory as low as 500 μM. Human supplementation studies (16, 17) demonstrate that following a dose of caffeine in the range of 5–6 mg/kg, caffeine can reach a plasma concentration of ~30 μM. With oral dosing, this concentration would be much higher in the portal vein and therefore chronic exposure to caffeine dosing could conceivably interfere with L-carnitine accumulation in the liver and potentially the skeletal muscle. Further work should explore the influence that caffeine and other AMPK activators have on L-carnitine balance in metabolically active tissues.

In conclusion, this study is the first to show that insulin in a relative high dose modestly increases L-carnitine transport into a skeletal muscle cell line. Additionally, we demonstrate that AMPK activators do not drive L-carnitine uptake but rather substantially inhibit uptake. While we were unable to confirm whether AMPK was required for inhibition by these compounds, we were able to demonstrate that the inhibitory effects of caffeine are independent of calcium release and partially sensitive to the AMPK inhibitor Compound C.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

A.S., S.J., K.R.W., J.D.P., I.G., T.W., G.D., N.B., S.G., and D.L.H. conceived and designed research; A.S., S.J., K.R.W., J.D.P., G.D., N.B., and D.L.H. performed experiments; A.S., S.J., K.R.W., J.D.P., I.G., T.W., G.D., N.B., and D.L.H. analyzed data; A.S., S.J., K.R.W., J.D.P., I.G., T.W., S.G., and D.L.H. interpreted results of experiments; K.R.W., J.D.P., T.W., and D.L.H. prepared figures.A.S., S.G., and D.L.H. drafted manuscript; A.S., S.J., K.R.W., J.D.P., I.G., T.W., G.D., N.B., S.G., and D.L.H. edited and revised

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REFERENCES

1. **Barnard RJ, Youngren JF.** Regulation of glucose transport in skeletal muscle. *FASEB J* 6: 3238–3244, 1992.
2. **Benziane B, Björnholm M, Pirkmajer S, Austin RL, Kotova O, Viollet B, Zierath JR, Chibalin AV.** Activation of AMP-activated protein kinase stimulates Na^+,K^+-ATPase activity in skeletal muscle cells. *J Biol Chem* 287: 23451–23463, 2012. doi:10.1074/jbc.M111.331926.
3. **Bergeron R, Previs SF, Cline GW, Perret P, Russell RR III, Young LH, Shulman GI.** Effect of 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside infusion on in vivo glucose and lipid metabolism in lean and obese Zucker rats. *Diabetes* 50: 1076–1082, 2001. doi:10.2337/diabetes.50.5.1076.
4. **Bergeron R, Russell RR III, Young LH, Ren JM, Marcucci M, Lee A, Shulman GI.** Effect of AMPK activation on muscle glucose metabolism in conscious rats. *Am J Physiol Endocrinol Metab* 276: E938–E944, 1999.
5. **Bonen A, Han XX, Habets DD, Febbraio M, Glatz JF, Luiken JJ.** A null mutation in skeletal muscle FAT/CD36 reveals its essential role in insulin- and AICAR-stimulated fatty acid metabolism. *Am J Physiol Endocrinol Metab* 292: E1740–E1749, 2007. doi:10.1152/ajpendo.00579.2006.
6. **Brass EP.** Pharmacokinetic considerations for the therapeutic use of carnitine in hemodialysis patients. *Clin Ther* 17: 176–185, 1995. doi:10.1016/0149-2918(95)80017-4.
7. **Brown AE, Elstner M, Yeaman SJ, Turnbull DM, Walker M.** Does impaired mitochondrial function affect insulin signaling and action in cultured human skeletal muscle cells? *Am J Physiol Endocrinol Metab* 294: E97–E102, 2008. doi:10.1152/ajpendo.00267.2007.
8. **Cheng Z, Pang T, Gu M, Gao AH, Xie CM, Li JY, Nan FJ, Li J.** Berberine-stimulated glucose uptake in L6 myotubes involves both AMPK and p38 MAPK. *Biochim Biophys Acta* 1760: 1682–1689, 2006. doi:10.1016/j.bbagen.2006.09.007.
9. **Dzamko NL, Steinberg GR.** AMPK-dependent hormonal regulation of whole-body energy metabolism. *Acta Physiol (Oxf)* 196: 115–127, 2009. doi:10.1111/j.1748-1716.2009.01969.x.
10. **El-Hattab AW, Scaglia F.** Disorders of carnitine biosynthesis and transport. *Mol Genet Metab* 116: 107–112, 2015. doi:10.1016/j.ymgme.2015.09.004.
11. **Flaten GE, Dhanikula AB, Luthman K, Brandl M.** Drug permeability across a phospholipid vesicle based barrier: a novel approach for studying passive diffusion. *Eur J Pharm Sci* 27: 80–90, 2006. doi:10.1016/j.ejps.2005.08.007.
12. **Fritz IB, Yue KT.** Long-chain carnitine acyltransferase and the role of acylcarnitine derivatives in the catalytic increase of fatty acid oxidation induced by carnitine. *J Lipid Res* 4: 279–288, 1963.
13. **Galloway SD, Craig TP, Cleland SJ.** Effects of oral L-carnitine supplementation on insulin sensitivity indices in response to glucose feeding in lean and overweight/obese males. *Amino Acids* 41: 507–515, 2011. doi:10.1007/s00726-010-0770-5.
14. **Galloway SD, Broad EM.** Oral L-carnitine supplementation and exercise metabolism. *Monatsh Chem* 136: 1391–1410, 2005. doi:10.1007/s00706-005-0288-9.
15. **Georges B, Le Borgne F, Galland S, Isoir M, Ecosse D, Grand-Jean F, Demarquoy J.** Carnitine transport into muscular cells. Inhibition of transport and cell growth by mildronate. *Biochem Pharmacol* 59: 1357–1363, 2000. doi:10.1016/S0006-2952(00)00265-3.
16. **Graham TE, Spriet LL.** Metabolic, catecholamine, and exercise performance responses to various doses of caffeine. *J Appl Physiol (1985)* 78: 867–874, 1995.
17. **Greer F, Friars D, Graham TE.** Comparison of caffeine and theophylline ingestion: exercise metabolism and endurance. *J Appl Physiol (1985)* 89: 1837–1844, 2000.
18. **Hardie DG, Ross FA, Hawley SA.** AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nat Rev Mol Cell Biol* 13: 251–262, 2012. doi:10.1038/nrm3311.
19. **Hawley SA, Pan DA, Mustard KJ, Ross L, Bain J, Edelman AM, Frenguelli BG, Hardie DG.** Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. *Cell Metab* 2: 9–19, 2005. doi:10.1016/j.cmet.2005.05.009.
20. **Hayashi T, Hirshman MF, Fujii N, Habinowski SA, Witters LA, Goodyear LJ.** Metabolic stress and altered glucose transport: activation of AMP-activated protein kinase as a unifying coupling mechanism. *Diabetes* 49: 527–531, 2000. doi:10.2337/diabetes.49.4.527.
21. **Hoehn KL, Turner N, Swarbrick MM, Wilks D, Preston E, Phua Y, Joshi H, Furler SM, Larance M, Hegarty BD, Leslie SJ, Pickford R, Hoy AJ, Kraegen EW, James DE, Cooney GJ.** Acute or chronic upregulation of mitochondrial fatty acid oxidation has no net effect on whole-body energy expenditure or adiposity. *Cell Metab* 11: 70–76, 2010. doi:10.1016/j.cmet.2009.11.008.
22. **Hyde R, Peyrollier K, Hundal HS.** Insulin promotes the cell surface recruitment of the SAT2/ATA2 system A amino acid transporter from an endosomal compartment in skeletal muscle cells. *J Biol Chem* 277: 13628–13634, 2002. doi:10.1074/jbc.M108609200.
23. **Jakobsen SN, Hardie DG, Morrice N, Tornqvist HE.** 5′-AMP-activated protein kinase phosphorylates IRS-1 on Ser-789 in mouse C2C12 myotubes in response to 5-aminoimidazole-4-carboxamide riboside. *J Biol Chem* 276: 46912–46916, 2001. doi:10.1074/jbc.C100483200.
24. **Jonker JW, Schinkel AH.** Pharmacological and physiological functions of the polyspecific organic cation transporters: OCT1, 2, and 3 (SLC22A1-3). *J Pharmacol Exp Ther* 308: 2–9, 2004. doi:10.1124/jpet.103.053298.
25. **Kim WS, Lee YS, Cha SH, Jeong HW, Choe SS, Lee MR, Oh GT, Park HS, Lee KU, Lane MD, Kim JB.** Berberine improves lipid dysregulation in obesity by controlling central and peripheral AMPK activity. *Am J Physiol Endocrinol Metab* 296: E812–E819, 2009. doi:10.1152/ajpendo.90710.2008.
26. **Kimura H, Takeda M, Narikawa S, Enomoto A, Ichida K, Endou H.** Human organic anion transporters and human organic cation transporters mediate renal transport of prostaglandins. *J Pharmacol Exp Ther* 301: 293–298, 2002. doi:10.1124/jpet.301.1.293.
27. **Krause T, Gerbershagen MU, Fiege M, Weisshorn R, Wappler F.** Dantrolene–a review of its pharmacology, therapeutic use and new developments. *Anaesthesia* 59: 364–373, 2004. doi:10.1111/j.1365-2044.2004.03658.x.
28. **Lee YS, Kim WS, Kim KH, Yoon MJ, Cho HJ, Shen Y, Ye JM, Lee CH, Oh WK, Kim CT, Hohnen-Behrens C, Gosby A, Kraegen EW, James DE, Kim JB.** Berberine, a natural plant product, activates AMP-activated protein kinase with beneficial metabolic effects in diabetic and insulin-resistant states. *Diabetes* 55: 2256–2264, 2006. doi:10.2337/db06-0006.
29. **Li FY, El-Hattab AW, Bawle EV, Boles RG, Schmitt ES, Scaglia F, Wong LJ.** Molecular spectrum of SLC22A5 (OCTN2) gene mutations detected in 143 subjects evaluated for systemic carnitine deficiency. *Hum Mutat* 31: E1632–E1651, 2010. doi:10.1002/humu.21311.
30. **Liang KW, Yin SC, Ting CT, Lin SJ, Hsueh CM, Chen CY, Hsu SL.** Berberine inhibits platelet-derived growth factor-induced growth and migration partly through an AMPK-dependent pathway in vascular smooth muscle cells. *Eur J Pharmacol* 590: 343–354, 2008. doi:10.1016/j.ejphar.2008.06.034.
31. **Liu LZ, Cheung SC, Lan LL, Ho SK, Xu HX, Chan JC, Tong PC.** Berberine modulates insulin signaling transduction in insulin-resistant cells. *Mol Cell Endocrinol* 317: 148–153, 2010. doi:10.1016/j.mce.2009.12.027.
32. **Luiken JJ, Koonen DP, Willems J, Zorzano A, Becker C, Fischer Y, Tandon NN, Van Der Vusse GJ, Bonen A, Glatz JF.** Insulin stimulates long-chain fatty acid utilization by rat cardiac myocytes through cellular redistribution of FAT/CD36. *Diabetes* 51: 3113–3119, 2002. doi:10.2337/diabetes.51.10.3113.
33. **Ma X, Egawa T, Kimura H, Karaike K, Masuda S, Iwanaka N, Hayashi T.** Berberine-induced activation of 5′-adenosine monophosphate-activated protein kinase and glucose transport in rat skeletal muscles. *Metabolism* 59: 1619–1627, 2010. doi:10.1016/j.metabol.2010.03.009.
34. **Martinuzzi A, Vergani L, Rosa M, Angelini C.** L-carnitine uptake in differentiating human cultured muscle. *Biochim Biophys Acta* 1095: 217–222, 1991. doi:10.1016/0167-4889(91)90102-4.
35. **McGlory C, White A, Treins C, Drust B, Close GL, Maclaren DP, Campbell IT, Philp A, Schenk S, Morton JP, Hamilton DL.** Application of the [γ-^{32}P] ATP kinase assay to study anabolic signaling in human skeletal muscle. *J Appl Physiol (1985)* 116: 504–513, 2014. doi:10.1152/japplphysiol.01072.2013.
36. **Mustard JA.** The buzz on caffeine in invertebrates: effects on behavior and molecular mechanisms. *Cell Mol Life Sci* 71: 1375–1382, 2014. doi:10.1007/s00018-013-1497-8.
37. **Ojuka EO, Jones TE, Nolte LA, Chen M, Wamhoff BR, Sturek M, Holloszy JO.** Regulation of GLUT4 biogenesis in muscle: evidence for

involvement of AMPK and Ca^{2+}. *Am J Physiol Endocrinol Metab* 282: E1008–E1013, 2002. doi:10.1152/ajpendo.00512.2001.

38. **Oshima R, Yamada M, Kurogi E, Ogino Y, Serizawa Y, Tsuda S, Ma X, Egawa T, Hayashi T.** Evidence for organic cation transporter-mediated metformin transport and 5′-adenosine monophosphate-activated protein kinase activation in rat skeletal muscles. *Metabolism* 64: 296–304, 2015. doi:10.1016/j.metabol.2014.10.037.
39. **Patel N, Khayat ZA, Ruderman NB, Klip A.** Dissociation of 5′ AMP-activated protein kinase activation and glucose uptake stimulation by mitochondrial uncoupling and hyperosmolar stress: differential sensitivities to intracellular Ca^{2+} and protein kinase C inhibition. *Biochem Biophys Res Commun* 285: 1066–1070, 2001. doi:10.1006/bbrc.2001.5275.
40. **Pochini L, Scalise M, Galluccio M, Indiveri C.** OCTN cation transporters in health and disease: role as drug targets and assay development. *J Biomol Screen* 18: 851–867, 2013. doi:10.1177/1087057113493006.
41. **Pressman BC.** Biological applications of ionophores. *Annu Rev Biochem* 45: 501–530, 1976. doi:10.1146/annurev.bi.45.070176.002441.
42. **Rodgers RL, Christe ME, Tremblay GC, Babson JR, Daniels T.** Insulin-like effects of a physiologic concentration of carnitine on cardiac metabolism. *Mol Cell Biochem* 226: 97–105, 2001. doi:10.1023/A:1012793924469.
43. **Russell RR III, Bergeron R, Shulman GI, Young LH.** Translocation of myocardial GLUT-4 and increased glucose uptake through activation of AMPK by AICAR. *Am J Physiol Heart Circ Physiol* 277: H643–H649, 1999.
44. **Shannon CE, Nixon AV, Greenhaff PL, Stephens FB.** Protein ingestion acutely inhibits insulin-stimulated muscle carnitine uptake in healthy young men. *Am J Clin Nutr* 103: 276–282, 2016. doi:10.3945/ajcn.115.119826.
45. **Shibbani K, Fahed AC, Al-Shaar L, Arabi M, Nemer G, Bitar F, Majdalani M.** Primary carnitine deficiency: novel mutations and insights into the cardiac phenotype. *Clin Genet* 85: 127–137, 2014. doi:10.1111/cge.12112.
46. **Spriet LL, Perry CG, Talanian JL.** Legal pre-event nutritional supplements to assist energy metabolism. *Essays Biochem* 44: 27–43, 2008. doi:10.1042/bse0440027.
47. **Steinberg GR, Smith AC, Van Denderen BJ, Chen Z, Murthy S, Campbell DJ, Heigenhauser GJ, Dyck DJ, Kemp BE.** AMP-activated protein kinase is not down-regulated in human skeletal muscle of obese females. *J Clin Endocrinol Metab* 89: 4575–4580, 2004. doi:10.1210/jc.2004-0308.
48. **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* 581: 431–444, 2007. doi:10.1113/jphysiol.2006.125799.
49. **Stephens FB, Constantin-Teodosiu D, Laithwaite D, Simpson EJ, Greenhaff PL.** An acute increase in skeletal muscle carnitine content alters fuel metabolism in resting human skeletal muscle. *J Clin Endocrinol Metab* 91: 5013–5018, 2006. doi:10.1210/jc.2006-1584.
50. **Stephens FB, Constantin-Teodosiu D, Laithwaite D, Simpson EJ, Greenhaff PL.** Insulin stimulates L-carnitine accumulation in human skeletal muscle. *FASEB J* 20: 377–379, 2006. doi:10.1096/fj.05-4985fje.
51. **Stephens FB, Evans CE, Constantin-Teodosiu D, Greenhaff PL.** Carbohydrate ingestion augments L-carnitine retention in humans. *J Appl Physiol (1985)* 102: 1065–1070, 2007. doi:10.1152/japplphysiol.01011.2006.
52. **Stephens FB, Wall BT, Marimuthu K, Shannon CE, Constantin-Teodosiu D, Macdonald IA, Greenhaff PL.** Skeletal muscle carnitine loading increases energy expenditure, modulates fuel metabolism gene networks and prevents body fat accumulation in humans. *J Physiol* 591: 4655–4666, 2013. doi:10.1113/jphysiol.2013.255364.
53. **Stephenson DG.** Caffeine – a valuable tool in excitation-contraction coupling research. *J Physiol* 586: 695–696, 2008. doi:10.1113/jphysiol.2007.148270.
54. **Sun S, Wang K, Lei H, Li L, Tu M, Zeng S, Zhou H, Jiang H.** Inhibition of organic cation transporter 2 and 3 may be involved in the mechanism of the antidepressant-like action of berberine. *Prog Neuropsychopharmacol Biol Psychiatry* 49: 1–6, 2014. doi:10.1016/j.pnpbp.2013.11.005.
55. **Tamai I, Yabuuchi H, Nezu J, Sai Y, Oku A, Shimane M, Tsuji A.** Cloning and characterization of a novel human pH-dependent organic cation transporter, OCTN1. *FEBS Lett* 419: 107–111, 1997. doi:10.1016/S0014-5793(97)01441-5.
56. **Todesco L, Bur D, Brooks H, Török M, Landmann L, Stieger B, Krähenbühl S.** Pharmacological manipulation of L-carnitine transport into L6 cells with stable overexpression of human OCTN2. *Cell Mol Life Sci* 65: 1596–1608, 2008. doi:10.1007/s00018-008-8065-7.
57. **Wall BT, Stephens FB, Constantin-Teodosiu D, Marimuthu K, Macdonald IA, Greenhaff PL.** Chronic oral ingestion of L-carnitine and carbohydrate increases muscle carnitine content and alters muscle fuel metabolism during exercise in humans. *J Physiol* 589: 963–973, 2011. doi:10.1113/jphysiol.2010.201343.
58. **Wu MN, Ho K, Crocker A, Yue Z, Koh K, Sehgal A.** The effects of caffeine on sleep in Drosophila require PKA activity, but not the adenosine receptor. *J Neurosci* 29: 11029–11037, 2009. doi:10.1523/JNEUROSCI.1653-09.2009.
59. **Xu D, You G.** *Rethinking the regulation of L-carnitine transport in skeletal muscle cells*. Focus on "Multiple AMPK activators inhibit L-carnitine uptake in C2C12 skeletal muscle myotubes". *Am J Physiol Cell Physiol* [Epub ahead of print, 2017]. doi:10.1152/ajpcell.00093.2017.