Multiple AMPK activators inhibit L-Carnitine uptake in C_2C_{12} skeletal muscle myotubes.

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Abstract

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 hypothesised that AMPK activating compounds and insulin would increase L-Carnitine uptake in C_2C_{12} 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 100nM. However, L-Carnitine uptake was modestly increased at a dose of 150nM insulin. A range of AMPK activators that increase intracellular calcium content [caffeine (10mM, 5mM, 1mM, 0.5mM), A23187 (10μM)], inhibit mitochondrial function [Sodium Azide (75μM), Rotenone (1μM), Berberine (100μM), DNP (500μM)] or directly activate AMPK [AICAR (250μM)] were assessed for their ability to regulate L-Carnitine uptake. All compounds tested significantly inhibited L-Carnitine uptake. Inhibition by caffeine was not dantrolene (10μM) 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 (10μM) to rescue the effect of caffeine. Compound C offered a partial rescue of L-Carnitine uptake with 0.5mM 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.

Introduction

L-Carnitine [3-Hydroxy-4-(trimethylazaniumyl) butanoate] is a dipeptide compound which acts as a co-factor 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. 95% 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 hypoglycaemia respectively (10, 45). Low L-Carnitine content is particularly damaging to the heart and leads to a compromised ability to utilise 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); whilst oral L-Carnitine supplementation alters whole body glucose handling under an OGTT (13). Furthermore, Stephens *et al* (2013) 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 (52). 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 (80g 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.

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, that 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:AMP ratio (18). A decrease in the ATP: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: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 characterised 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 hypothesised that insulin and a range of AMPK activating compounds would enhance the transport of L-Carnitine into C_2C_{12} skeletal muscle myotubes. Contrary to our hypothesis however, we find that insulin had only a modest effect on L-Carnitine uptake at 150nM, and all AMPK activating reagents inhibited L-Carnitine uptake. These data have wider implications for the pharmacological treatment of L-Carnitine deficiency disorders and optimising L-Carnitine accumulation in skeletal muscle to enhance metabolism.

Methodology

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 lab consumables were sourced from Fisher Scientific unless otherwise stated.

Cell culture

C_2C_{12} myoblasts (ATCC) were cultured in 6 or 12 well plates and maintained in a growth media containing High Glucose Dulbecco's Modified Eagle Media (DMEM), 20% Fetal Bovine Serum (FBS) and a 1% Pen/Strep. Once 80-100% confluent the cells were differentiated using a Differentiating media containing High Glucose DMEM, 2% Donor Horse Serum and 1% Pen/Strep. Experiments were carried out once cells were fully differentiated into myotubes (3-5 days post differentiation).

Substrate transport assays

Cells were placed in a serum free media for 3 hours prior to the experiment. Post serum starve the cells were exposed to a tritiated L-carnitine buffer [100μM (or 0.1μM for insulin stimulation) at 80μCi/μmol] and each treatment was completed with the addition of Insulin (100nM and 150nM) or one of the following AMPK activators; AICAR [250μM (previously shown to activate AMPK at 500μM) (23)], 2, 4- Dinitrophenol (DNP) [500μM (activates AMPK in L6 myotubes at 500μM) (39)], Rotenone [1μM (previously demonstrated to activate AMPK in the low μM range) (20)], Berberine [100μM (30)], Caffeine [5mM (37)], A23187 [10μM (19)] and Sodium Azide [75μM (7)]. Following 3 hour 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 Ltd, Chesterfield, UK) for scintillation counting (United Technologies Packard 2200CA TriCarb). Uptake data were normalised back to protein content as assessed by Bradford assays.

For glucose uptakes cells were serum starved in serum and amino acid free PBS + 5mM glucose for 3 hr. After the 3 hr serum starve cells were stimulated with or without 100nM insulin for 30mins. Following the 30min insulin stimulation glucose uptake assays were carried out using a buffer containing Tritiated 2-Deoxyglucose (10µm at 0.66µCi/ml) for 10mins 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 Ltd, Chesterfield, UK) for scintillation counting (United Technologies Packard 2200CA TriCarb). Uptake data were normalised 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, Germany). 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; 1:1000; Abcam, Cambridge), 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 C_2C_{12} myoblasts were plated onto glass coverslips and then differentiated to myotubes as described previously. Differentiated cells were then loaded with 3µM of the calcium-sensitive dye, Fura-2 (Sigma Aldrich, Dorset, UK) in Locke's buffer medium [154mM NaCl, 4mM $NaHCO_3$, 5mM KCl, 2.3mM $CaCl_2$,1mM $MgCl_2$, 5mM glucose, 10mM 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 x3 in Locke's buffer and the coverslips placed into a microscope chamber containing Locke's buffer. The cells were then illuminated with an ultra high point intensity 75-watt xenon arc lamp (Optosource, Cairn Research, Faversham, Kent, UK) and imaged using a Nikon Diaphot inverted microscope equipped with a Nikon x40 oil immersion Fluor objective lens (NA = 1.3) and a monochromator (Optoscan, Cairn Research), which was used to alternate the excitation

wavelength between 340/380 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 Corp., Downing, PA) was used for the control of the monochromator, CCD camera, and for processing of the cell image data. 5mM Caffeine was added after 60 secs using a perfusion system. To measure the effect of dantrolene, the cells were preincubated for 20 mins with 10μM dantrolene [demonstrated to be effective at inhibiting calcium release in L6-myotubes (37)] prior to perfusion of 5mM caffeine. Ratiometric images captured at 2 sec intervals were then analysed using MetaFluor software. Changes in calcium were expressed as fold changes over normalised basal calcium for each cell. Data was collected for 23-33 cells over 3 separate experiments and statistical difference between mean maximal caffeine-induced calcium responses in the presence and absence of dantrolene analysed using a 2-tailed unpaired t-test using Excel, with significance determined as $p<0.05$.

Primers for PCR analysis were designed from reference sequences using Primer 3 (ref). RNA was extracted from C2C12 cells using Trizol reagent (Invitrogen) following standard protocols. 1ug of 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 8ul 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 -20ºC before use. Amplification of gene products was carried out using the Fermentas PCR kit (Fermentas, Thermo-Scientific, UK). Briefly 1ul of cDNA was combined with 12.5ul supplied PCR master mix, 1ul of forward and reverse primer each at 10μM and 9.5ul ddH2O. The reactions conditions were 95ºC for 1min, 35 cycles of 95ºC for 30s, 50ºC for 30s and 72ºC for 60s followed by a final 5 mins at 72ºC. Products were visualised by agarose gel electrophoresis in 1% agarose gel. Electrophoresis was carried out for 40 mins at 100V. Bands were imaged with a BioRad gel doc system (Biorad, UK)

Statistics

Unless otherwise stated, Figures and Statistical analyses were carried out using the Graph Pad 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

C_2C_{12} myotubes express OCTN1/2/3

Previous work has shown that primary human myotubes and C_2C_{12} myoblasts and myotubes transport L-Carnitine (15, 34). However, to ensure that our C_2C_{12} model would be a useful tool to study L-Carnitine transport we first assessed if the OCTN family of transporters that shuttle L-Carnitine into the cell were present in the C_2C_{12} 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 (Figure1A). To confirm expression at the protein level, we carried out immunohistochemistry experiments with a pan-OCTN1/2/3 antibody and identified that C_2C_{12} 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. (Figure1B).

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 hypothesised 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 (Figure 2A and 2B). Despite the obvious insulin responsiveness of our cell line, we found that 100nM did not have a significant effect on L-Carnitine uptake, however 150nM insulin did induce a modest but significant increase in uptake (Figure 2C).

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 7 ‘AMPK activating compounds’ and determined their ability to induce AMPK activity in C_2C_{12} myotubes. All compounds tested significantly activated AMPK after a 30 minute stimulation period to varying degrees ranging from 2.2 ± 0.58 fold to 5.42 ± 0.48 fold (Figure 3B). Having confirmed that each compound significantly activates AMPK we next evaluated the effect of these compounds on L-Carnitine uptake (Figure 3A). Contrary to our hypothesis, treatment of C_2C_{12}

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 not an organic cation, we performed a substrate-saturation curve to assess if caffeine could be inhibiting uptake via competitive inhibition of transport, as expected the kinetic curve revealed that the predicted V_{max} was reduced indicating non-competitive inhibition (Figure 4A). Further analysis of inhibition across a range of substrate concentrations revealed a dose dependent effect of caffeine across all substrate concentrations tested (Figure 4B). To test the calcium dependence of the effects of caffeine we assessed the impact of 10μM 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 (Figure 4C). To test if a lower dose of caffeine could be reversed by dantrolene we halved the dose of caffeine to 5mM and again dantrolene did not rescue the uptake inhibition (Figure 4D). This is in spite of our findings that dantrolene substantially inhibited the appearance of calcium in response to caffeine stimulation (Figure 4E). We next assessed if the inhibitory effect was dependent upon AMPK by using the inhibitor Compound C. These data revealed a partial rescue of Carnitine transport at a relatively low dose of caffeine (0.5mM) but not at the higher dose of caffeine (5mM) 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 C_2C_{12} myotubes is associated with 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 non-physiological ranges (15, 34). Here we investigated the pharmacological regulation of L-Carnitine uptake in C_2C_{12} myotubes across these affinity ranges for L-Carnitine transport during 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 C_2C_{12} 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 hypothesised 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 oubain sensitive and dependent upon extracellular sodium (15). Therefore the 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 80g 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 (150nM vs 100nM) is utilized. Whilst in humans, insulin has not been shown to affect L-Carnitine uptake but rather balance and accumulation, our data suggests that perhaps if insulin achieves a high enough concentration it could modify skeletal muscle uptake.

Analogues 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 sub-family of OCTN related transporters OCT2/3 with an IC50 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 100μM Berberine could be due to competitive inhibition. Analysis of over expressed 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 characterised, 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 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 upon glycolysis and anaerobic respiration would lead to an acidification of the cellular environment which could cause

293 an inhibition of L-Carnitine transport which requires an optimal pH range to function fully (15). One
294 interesting aspect however, is that OCTN1 has been identified as having a nucleotide-binding site and
295 displays transport function inhibited by ATP depletion by glycolytic inhibitors and mitochondrial
296 toxins (55). These data suggest that transport of L-Carnitine by OCTN1, at least, could be inhibited
297 directly by reductions in ATP. This could also be a potential mechanism by which the AMP mimetic
298 AICAR could work. If ZMP instead of ATP occupies the nucleotide-binding site it may impair
299 function. However, it must be stated that the nucleotide dependency of OCTN transporters has never
300 been confirmed nor has the ability of AMP to regulate function.

301 All of the compounds mentioned above, must be transported into the cell to act on various
302 intracellular molecules or directly on the mitochondria. Due to the broad range of molecules that
303 could act as substrates (56) for the OCTN transport mechanisms that transport L-Carnitine it is
304 possible that the above compounds could interfere as competitive inhibitors of transport. The calcium
305 release compounds however, act either by increasing the permeability of the plasma membrane to
306 Ca2+ (41) or by inducing Ca2+ release from the sarcoplasmic reticulum (53). Both are lipophilic
307 molecules capable of diffusing across the membrane independently of active transport mechanisms
308 (11, 41) and therefore competitive inhibition is unlikely to be the mode of action. Both molecules
309 significantly activated AMPK by ~2fold and both molecules inhibited L-Carnitine uptake by ~50%
310 (A23187) and ~80% (caffeine). Dantrolene (10μM) which inhibits the release of calcium from
311 intracellular stores (27) significantly inhibited calcium release with 5mM caffeine, surprisingly it did
312 not rescue the inhibitory effect of caffeine on L-Carnitine uptake. These data suggested that the
313 influence of caffeine, at least, was independent of changes in Ca2+. We therefore assessed the impact
314 of caffeine on L-Carnitine transport kinetics, which confirmed that caffeine was non-competitively
315 inhibiting transport. We next tested the dependency of the inhibitory effects of caffeine on AMPK
316 with the AMPK inhibitor Compound C (10μM). Compound C did not rescue the effect of caffeine at
317 high doses (5mM) but did partially recover uptake at a much lower dose of caffeine (0.5mM)
318 suggesting that the inhibition of uptake was at least partially dependent upon 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 500μM. Human supplementation studies (16, 17) demonstrate that following a dose of caffeine in the range of 5-6mg/kg, caffeine can reach a plasma concentration of ~30μM. 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.

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504 Table 1. Primer details for *M. 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

505

506

Figure Legends.

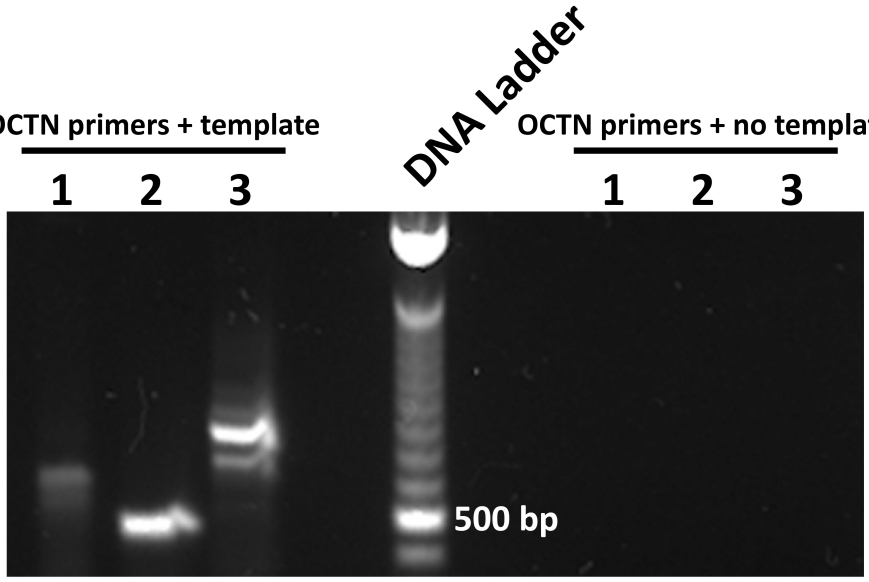
Figure 1. Expression of Carnitine transporter isoforms in C_2C_{12} myotubes. PCR amplification demonstrates the expression of the three OCTN isoforms in C2C12 myotubes (A). Immunohistochemical analysis with a pan OCTN1/2/3 antibody demonstrates expression at the protein level (B). Blue indicates DAPI staining of the nucleus and red indicates OCTN1/2/3.

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

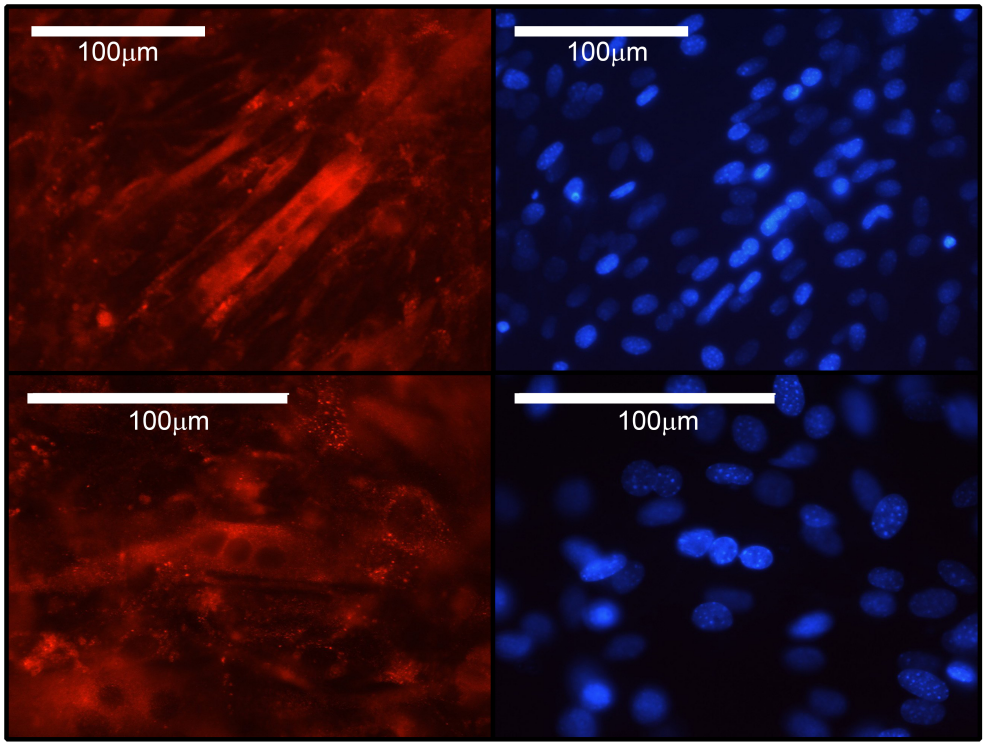
Figure 3. AMPK activators inhibit L-Carnitine transport. Cells were treated with AICAR (250μM), Azide (75μM), Berberine (100μM), DNP (500μM), Caffeine (10mM), A23187 (10μM) and L-Carnitine transport determined (A) and AMPK activity assessed (B). Compounds were present throughout the uptake assay and data are normalised to each respective control. *indicates significantly different from baseline.

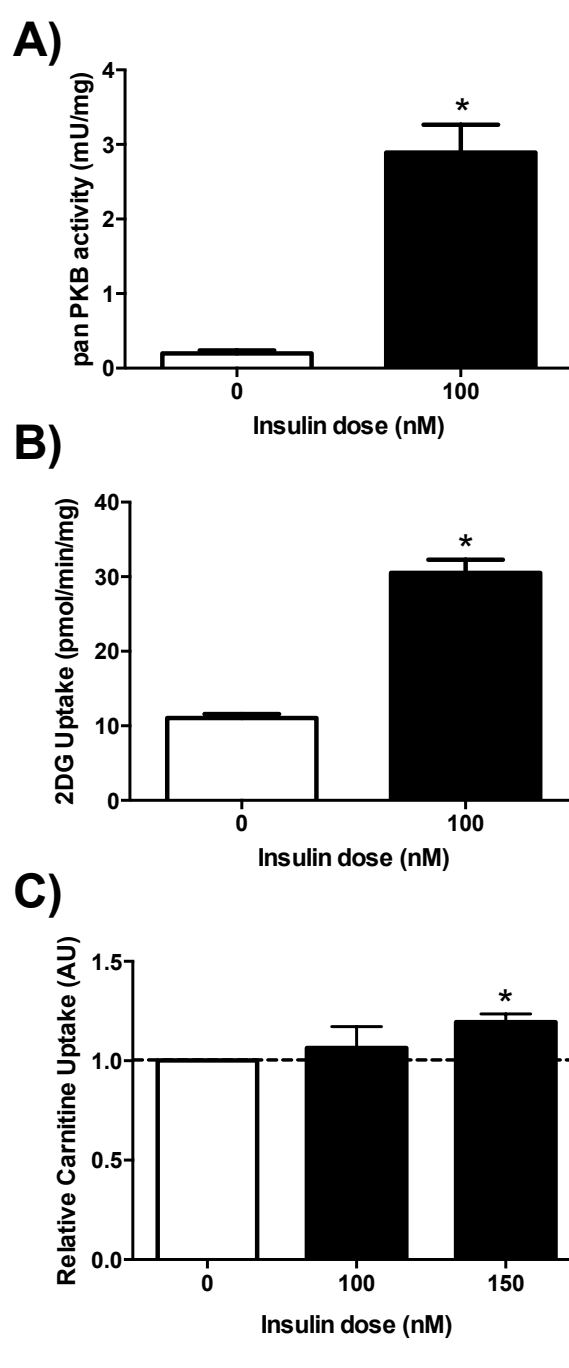
Figure 4. Caffeine inhibits L-Carnitine transport independently of calcium release. Uptake kinetics were assessed with increasing amounts of tracee in the presence or absence of 5mM caffeine (A). Inhibition by caffeine was assessed with 1 or 5mM caffeine at 100, 50, 10 or 0.1μM L-Carnitine (B). Cells were pre-treated with dantrolene (10μM) prior to the addition of caffeine during the uptake and calcium imaging experiments. Caffeine (10mM) or A23187 (10μM) were added following a 10min pre-incubation with dantrolene (10μM) and L-Carnitine uptake assessed. Data are represented as % of baseline (C). L-Carnitine uptake was assessed with 0mM, 5mM caffeine and 10mM caffeine with or without 10μM dantrolene (D). Calcium release was assessed as described in the methods with 5mM caffeine with or without 10μM dantrolene (E). Uptake of L-Carnitine was assessed with 0.5mM or 5mM caffeine in the presence or absence of 10μM compound C (F). * indicates significantly different from baseline, # indicates significantly different from 1mM caffeine. α indicates significantly different from 5mM caffeine at peak, δ indicates significantly different from 5mM caffeine at 60mins.

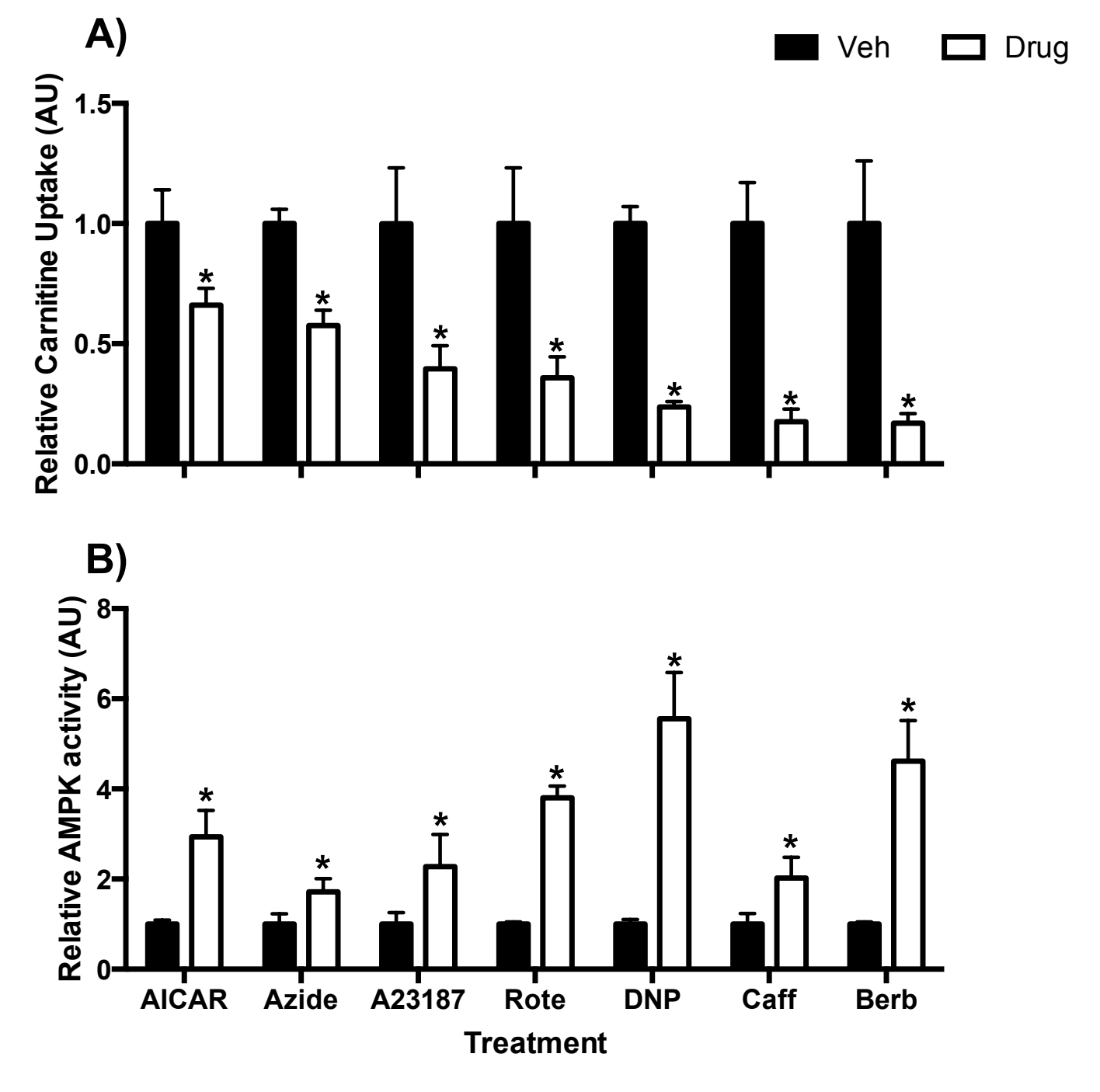
A)

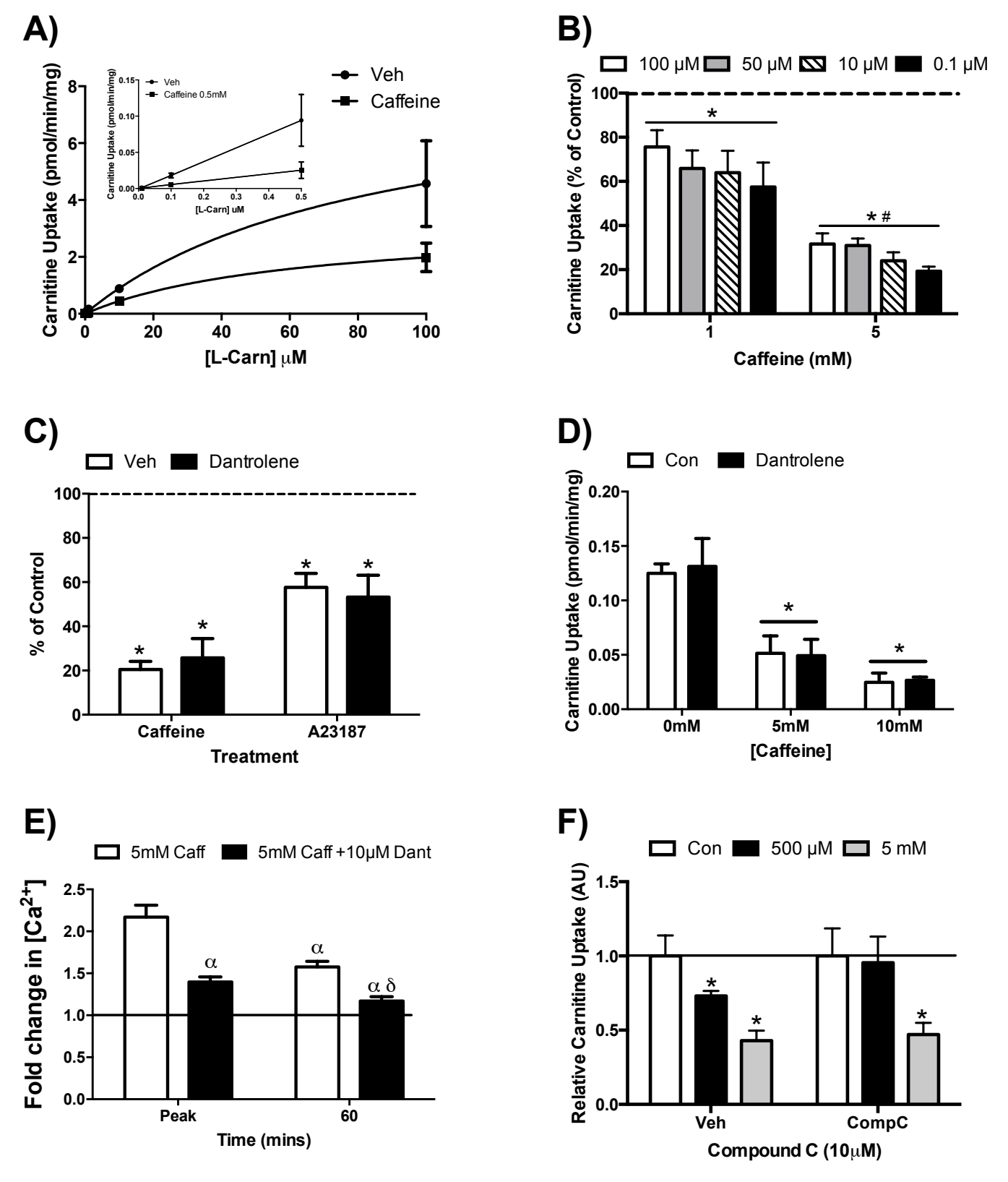

OCTN primers + template
1
2
3
DNA Ladder
OCTN primers + no template
1
2
3
500 bp

B)


100µm
100µm
100µm
100µm
40x
63x


A)
pan PKB activity (mU/mg)
4
3
2
1
0
*
0
100
Insulin dose (nM)
B)
2DG Uptake (pmol/min/mg)
40
30
20
10
0
*
0
100
Insulin dose (nM)
C)
Relative Carnitine Uptake (AU)
1.5
1.0
0.5
0.0
*
0
100
150
Insulin dose (nM)


A)
Veh
Drug
Relative Carnitine Uptake (AU)
1.5
1.0
0.5
0.0
B)
Relative AMPK activity (AU)
8
6
4
2
0
AICAR
Azide
A23187
Rote
DNP
Caff
Berb
Treatment


A)
Veh
Caffeine
Carnitine Uptake (pmol/min/mg)
[L-Carn] μM
Veh
Caffeine 0.5mM
Carnitine Uptake (pmol/min/mg)
[L-Carn] uM
B)
100 μM
50 μM
10 μM
0.1 μM
Carnitine Uptake (% of Control)
Caffeine (mM)
C)
Veh
Dantrolene
% of Control
Caffeine
A23187
Treatment
D)
Con
Dantrolene
Carnitine Uptake (pmol/min/mg)
0mM
5mM
10mM
[Caffeine]
E)
5mM Caff
5mM Caff +10μM Dant
Fold change in [Ca2+]
Peak
60
Time (mins)
F)
Con
500 μM
5 mM
Relative Carnitine Uptake (AU)
Veh
CompC
Compound C (10μM)