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Carnitine protects the nematode *Caenorhabditis elegans* from glucose-induced reduction of survival depending on the nuclear hormone receptor DAF-12

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ABSTRACT

Besides its function in transport of fatty acids into mitochondria in order to provide substrates for β-oxidation, carnitine has been shown to affect also glucose metabolism and to inhibit several mechanisms associated with diabetic complications. In the present study we used the *mev-1* mutant of the nematode *Caenorhabditis elegans* fed on a high glucose concentration in liquid media as a diabetes model and tested the effects of carnitine supplementation on their survival under heat-stress. Carnitine at 100 μM completely prevented the survival reduction that was caused by the application of 10 mM glucose. RNA-interference for *sir-2.1*, a candidate genes mediating the effects of carnitine revealed no contribution of the sirtuin for the rescue of survival. Under *daf-12* RNAi rescue of survival by carnitine was abolished. RNA-interference for *γ-butyrobetaine hydroxylase 2*, encoding the key enzyme for carnitine biosynthesis did neither increase glucose toxicity nor prevent the rescue of survival by carnitine, suggesting that the effects of carnitine supplementation on carnitine levels were significant. Finally, it was demonstrated that neither the amount of lysosomes nor the proteasomal activity were increased by carnitine, excluding that protein degradation pathways, such as autophagy or proteasomal degradation, are involved in the protective carnitine effects.

In conclusion, carnitine supplementation prevents the reduction of survival caused by glucose in *C. elegans* in dependence on a nuclear hormone receptor which displays high homologies to the vertebrate peroxisomal proliferator activated receptors.

1. Introduction

L-carnitine or 3-hydroxy-4-N,N,N-trimethylaminobutyric acid is essential for the normal function of all tissues [1]. Its endogenous synthesis in mammals starts from lysosomal proteolysis and the threefold N-methylation of lysine in lysosomes especially of liver and kidney [2]. As a member of the carnitine shuttle system carnitine is essential for the translocation of long-chain fatty acids into mitochondria for β-oxidation [3]. Moreover, carnitine is also necessary for the efflux of acetyl and acyl groups out of the mitochondria in the case that substrate oxidation exceeds the energy demand, and therefore, accumulating acyl-CoA intermediates are converted back to acylcarnitines that are transported out of mitochondria and cells [1,4]. Interestingly, there appears to exist a link between carnitine deficiency in developing mitochondrial dysfunction and insulin resistance during states of chronic metabolic stress as is the case in obesity or aging [5,6]. In addition, the carnitine-dependent removal of mitochondrial acetyl-CoA releases the inhibition of pyruvate-dehydrogenase and enables thereby pyruvate utilization with the free mitochondrial CoA, allowing an increased oxidation rate, as was demonstrated in the heart [7] and also in peripheral tissues [8]. It is reasonable to suggest that the latter effects become prominent in cells which display an unrestricted glucose uptake in diabetes mellitus, such as endothelial cells [9], pericytes [10], neurons [11], and glomerular cells [12], and that carnitine supplementation might be required in order to adapt to the increased requirements. Indeed, previous studies have

Abbreviations: LTR, LysoTracker® Red; NGM, nematode growth medium; PPAR, peroxisome proliferator activated receptor; ROS, reactive oxygen species; RNAi, RNA-interference; Suc-LLVY-AMC, succinyl-leucyl-leucyl-valine-aminomethyl-coumarine.

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shown that carnitine supplementation in pharmacological doses improved glucose tolerance in humans and animals [1,13]. However, besides the described effects of carnitine on glucose and fatty acid metabolism, anti-oxidative activities, mitochondrial biogenesis but also adverse effects at higher doses have to be considered [14,15].

In previous studies we have shown that the *mev-1* mutant of the nematode *Caenorhabditis elegans* provides a valid model to study the molecular mechanisms of glucose-induced toxicity which finally was evaluated according to the survival of the nematodes under heat-stress [16]. Moreover, we have identified several mechanisms linked to proteostasis, the biological machinery controlling the folding and degradation thus the functionality of proteins within a cell, which has to be necessarily activated through quercetin [17] or plant extracts [18] in order to enable those interventions to compensate the survival reduction as caused by glucose.

In the present study we used the *C. elegans mev-1* mutant in order to investigate whether carnitine is able to prevent the reduction of survival caused by the application of glucose. RNA-interference (RNAi) was accomplished to assess the contribution of single candidate genes on those effects. Moreover, the formation of lysosomes was quantified and the chymotrypsin-like activities were estimated in order to unravel whether autophagosomal and proteasomal degradation pathways were involved in carnitine mediated effects, respectively.

2. Materials and methods

2.1. Reagents

SYTOX green nucleic acid stain was obtained from Life Technologies (Karlsruhe, Germany). L-Carnitine inner salt, Suc-LLVY-AMC and all other materials used were purchased from Sigma–Aldrich (Steinheim, Germany).

2.2. Strains and maintenance

C. elegans TK22 *mev-1(kn1)* and *E. coli* OP50 were obtained from *C. elegans* Genetics Center (CGC, University of Minnesota, USA). Nematodes were maintained on nematode growth medium (NGM) agar plates seeded with *E. coli* OP50 at 20 °C according to standard protocols [19]. For all experiments synchronous populations were used that were obtained by bleaching [20]. Synchronous nematodes were raised in liquid culture using NGM liquid blended with *E. coli* OP50 or an appropriate RNAi clone [20]. Carbenicillin was added to the NGM liquid in order to inactivate *E. coli*. A volume of 46 μL of NGM liquid was dispensed in each well of a 96-well microplate, to which 10 μL M9 buffer containing ten synchronized L1 larvae were added. L1 larvae were maintained shaking at 20 °C and reached the adult stage within 3 days. Nematodes were treated with effectors at the young adult stage for 48 h.

E. coli HT115 RNAi clones were purchased from Source Bioscience (Cambridge, UK) and included a negative control (L4440), *sir-2.1* (R11A8.4), *daf-12* (F11A1.3), and *ubq-1* (F25B5.4). The RNAi clone *gbh-2* (M05D6.7) was from *C. elegans* ORF-RNAi library [21].

2.3. Glucose and carnitine treatment

A 1 M glucose solution and a 10 mM carnitine solution were prepared in M9 buffer. This was diluted with M9 buffer for a 100 mM glucose working solution and a 1 mM carnitine working solution, respectively. When nematodes had reached the young adult stage, 7 μL of glucose working solution or in addition 7 μL of carnitine working solution were added to the media. M9 buffer was added in equivalent amounts to the control. After 48 h incubation, worms were prepared for measurement.

2.4. RNAi experiments

RNAi experiments were performed in liquid cultures as described [22]. In brief, expression of gene-specific dsRNA in the corresponding RNAi bacterial strain was induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 4 h at 37 °C. Subsequently bacteria cells were washed and resuspended in NGM liquid added with 40 μg/ml kanamycin to inactivate *E. coli* HT115. A volume of 10 μL M9-buffer containing 10 synchronized L1 larvae was dispensed into each well of a 96-well-plate to which 46 μL of *E. coli* HT115 NGM suspension were added. For preparations of nematodes in order to determine proteasomal activity ten-fold increased approaches were set up in 24-well-plates (Greiner Bio-One, Frickenhausen, Germany). In general, L1 larvae reached the adult stage within 3 days of incubation with agitation at 20 °C before they were treated with effectors for 48 h (see Section 2.3).

2.5. Quantitative real-time PCR

Total RNA was extracted from 10,000 worms using Trizol. One-step-real-time PCR reactions were performed in triplicate using 1 μl of RNA template (10 ng/μl), Brilliant II SYBR Green QRT-PCR Mastermix and appropriate primers in a CFXTM Real-Time PCR Detection System (Bio-Rad, München, Germany). Cycling conditions were 1 × [15 min 50 °C], 1 × [10 min 95 °C], 40 × [30 s 95 °C, 15 s 52 °C, 30 s 60 °C], 1 × [1 min 95 °C], 1 × [30 s 52 °C–95 °C]. Changes in target gene expression were calculated according to Pfaffl [23] using equation $2^{-\Delta\Delta CT}$. For determination of RNAi efficiency unique primer pairs (Eurofin MWG Operon, Ebersberg, Germany) recognizing only cDNA derived from endogenous mRNA were designed to avoid cross-reaction with genomic DNA and bacterially generated dsRNA and were as follows: *18S-rRNA* fw-5′-ATG GTT GCA AAG CTG AAA CT-3′, *18S-rRNA* rev-5′-TCC CGT GTT GAG TCA AAT TA-3′; *sir-2.1* fw-5′-GAC AAA GAA CAG AAA GTA CAA CCA G-3′, *sir-2.1* rev-5′- GGA GTG GCA CCA TCA TCA AG-3′; *daf-12* fw-5′- CAA CGT GGA TGA TAT GTT TG-3′, *daf-12* rev-5′-GAA GAA ACC GAA GAA CTC TA-3′; *ubq-1* fw-5′-GCG TCT TAT CTT TGC TGG-3′, *ubq-1* rev-5′-GAG CAC CAA GTG GAG AG-3′; *gbh-2* fw-5′-ACGGCGTAGAAGGAACATCA-3′, *gbh-2* rev-5′-AGGTTCGTCCTGAAA-GATGGA-3′. For each sample the fold change in the target gene was normalized to *18S rRNA* and relative to the expression in the vector control, which was fed on L4440 bacteria. Relative mRNA levels in nematodes cultured on RNAi bacteria were significantly lower ($p < 0.01$) than mRNA levels of the vector control and were below 0.25 for all genes tested.

2.6. Survival at 37 °C

Lifespan analysis under heat stress conditions (37 °C) was performed 48 h after the addition of effectors as previously described [24]. In brief, nematodes were washed off the wells with M9-buffer/Tween®20 into 15 ml tubes followed by additional three washing steps. In each well of a black 384-well low-volume microtiter plate (Greiner Bio-One, Frickenhausen, Germany) 6.5 μL M9-buffer/Tween®20 (1% v/v) solution were added. Subsequently one nematode was dispensed in 1 μL M9-buffer/Tween®20 into each well and mixed with 7.5 μL SYTOX green (final concentration 1 μM, Life Technologies, Karlsruhe, Germany). To prevent water evaporation the plates were sealed with Rotilab sealing film and covered with a lid (Greiner Bio-One, Frickenhausen, Germany). The induction of heat shock (37 °C) and the measurement of fluorescence were done with a Fluoroskan Ascent microtiter plate reader (Thermo

Labsystems, Bonn, Germany) every 30 min. To detect SYTOX green fluorescence, excitation wavelength was set to 485 nm and emission was measured at 538 nm.

To determine the survival time for each nematode an individual fluorescence curve was generated. Time of death was defined as one hour after an increase in fluorescence over the baseline level. From the individual times of death Kaplan–Meier survival curves were drawn.

2.7. Staining of lysosomes

The formation of lysosomes was quantified using the low molecular weight fluorescent dye LysoTracker® Red DND-99 (LTR; Life Technologies, Schwerte, Germany), that specifically accumulates in acidic organelles [25]. In brief, synchronized young adult nematodes were incubated with LTR (final concentration 2 μM) for 48 h simultaneously with the effectors. For epifluorescence microscopy (EVOS fl digital fluorescence microscope, AMG, Bothell, USA) worms were washed subsequently and anaesthetized by addition of 2 mM levamisole. LTR was visualized using the EVOS LED light cube RFP with an excitation at 531 ± 40 nm and an emission at 593 ± 40 nm. Images were taken at tenfold magnification and the quantification of fluorescence intensity was done using ImageJ (National Institute of Health, NIH).

2.8. Chymotrypsin-like proteasomal activity

For protein extraction nematodes were treated with lysis buffer (50 mM HEPES, 150 mM NaCl, 5 mM EDTA, 2 mM DTT) and freezed at −80 °C. After homogenization with Peqlab Precellys 24-Dual the concentration of solubilized proteins was determined by the Bio-Rad Protein Assay (Bio-Rad, Munich, Germany). Quantification of specific chymotrypsin-like proteasome activity was done by a fluorogenic peptide substrate assay as described [26]. In brief, the solubilized proteins were incubated with Suc-LLVY-AMC (final concentration 140 μM, Sigma–Aldrich Chemie, Steinheim, Germany) in proteasome activity assay buffer (50 mM HEPES pH7.4, 150 mM NaCl, 5 mM EDTA, 5 mM ATP) at room temperature. The fluorescence intensity was measured in triplicate over 3 h every 10 min with excitations at 355 nm and emissions at 460 nm, using a Fluoroskan Ascent microtiter plate reader (Thermo Labsystems, Bonn, Germany). To determine the specific proteasome activity, the average activity measured in the presence of proteasome inhibitor MG132 (final concentration 10 μM; Calbiochem, Merck, Darmstadt, Germany) was subtracted from activities due to overall fluorogenic peptide cleavage.

2.9. Calculations and statistics

Statistical analyses were performed with GraphPad Prism 5.0 software (GraphPad, La Jolla, CA, USA). Log rank test was performed to compare survival rates with p values <0.05 being significant. For group comparisons Analysis of Variance (ANOVA) was performed. Following ANOVA, differences between groups were determined by Bonferroni–Holm multiple comparison test. Results are presented as means ± SD.

3. Results

3.1. Carnitine extends the survival of glucose-treated mev-1 nematodes

As demonstrated previously, the addition of 10 mM glucose to the NGM reduced the survival time of *mev-1* nematodes significantly (Fig. 1). Here we show that carnitine at a concentration of 100 μM was able to prevent the survival reduction induced by 10 mM glucose completely (Fig. 1).

3.2. The effects of carnitine on survival are dependent on daf-12 but independent of sir-2.1 and carnitine biosynthesis

Since it was previously shown by us that the polyphenol quercetin compensates the glucose-induced reduction of survival depending on the transcription factors *sir-2.1* and *daf-12*, we tested here whether carnitine triggers the same signaling pathways as the polyphenol. Knockdown of the nuclear hormone receptor *daf-12* not only led to a high sensitivity of nematodes versus the addition of glucose but also prevented the survival extending effects of carnitine under simultaneous glucose exposure (Fig. 2A). In contrast, knockdown of *sir-2.1* was not able to prevent the survival extension caused by carnitine in *mev-1* mutants (Fig. 2B). In order to investigate whether carnitine synthesis contributes in any way to the survival extension caused by exogenous carnitine we performed RNAi for *gbh-2*, which encodes an ortholog of human gamma butyrobetaine hydroxylase 2. As shown in Fig. 2C, the knockdown of *gbh-2* did not prevent the carnitine-induced rescue of lifespan at 37 °C, indicating that carnitine biosynthesis does not contribute to the effects of exogenous carnitine.

3.3. Protein degradation pathways are not involved in the survival extension by carnitine

Since we have described previously that activation of the proteasome is crucial for the extension of survival in glucose-treated *mev-1* mutants by quercetin, we assessed in the present study the impact of carnitine on chymotrypsin-like proteasomal activity. This activity was reduced due to the addition of glucose but was further reduced by simultaneous carnitine application (Fig. 3A). Moreover, the extent of lysosomes as a measure of subsided autophagy, increased in the presence of glucose but was under these conditions not affected by applying in addition carnitine (Fig. 3B). In accordance with the resulting conclusion that the proteasome does not play a role for the carnitine effects on survival extension in the presence of glucose, *daf-12* RNAi did not display any influence on proteasomal activity (data not shown).

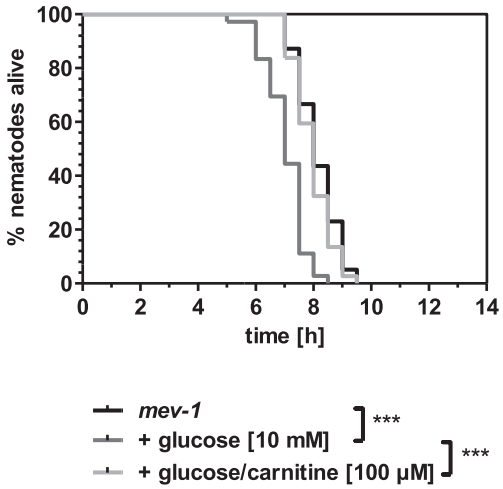

Fig. 1. Carnitine extends the survival of *C. elegans mev-1* mutants under glucose exposure. Treatment of nematodes with 10 mM glucose for 48 h decreases the survival of *mev-1* mutants under subsequently applied heat-stress at 37 °C. Additional application of 100 μM L-carnitine prevents the glucose-induced reduction of survival completely.

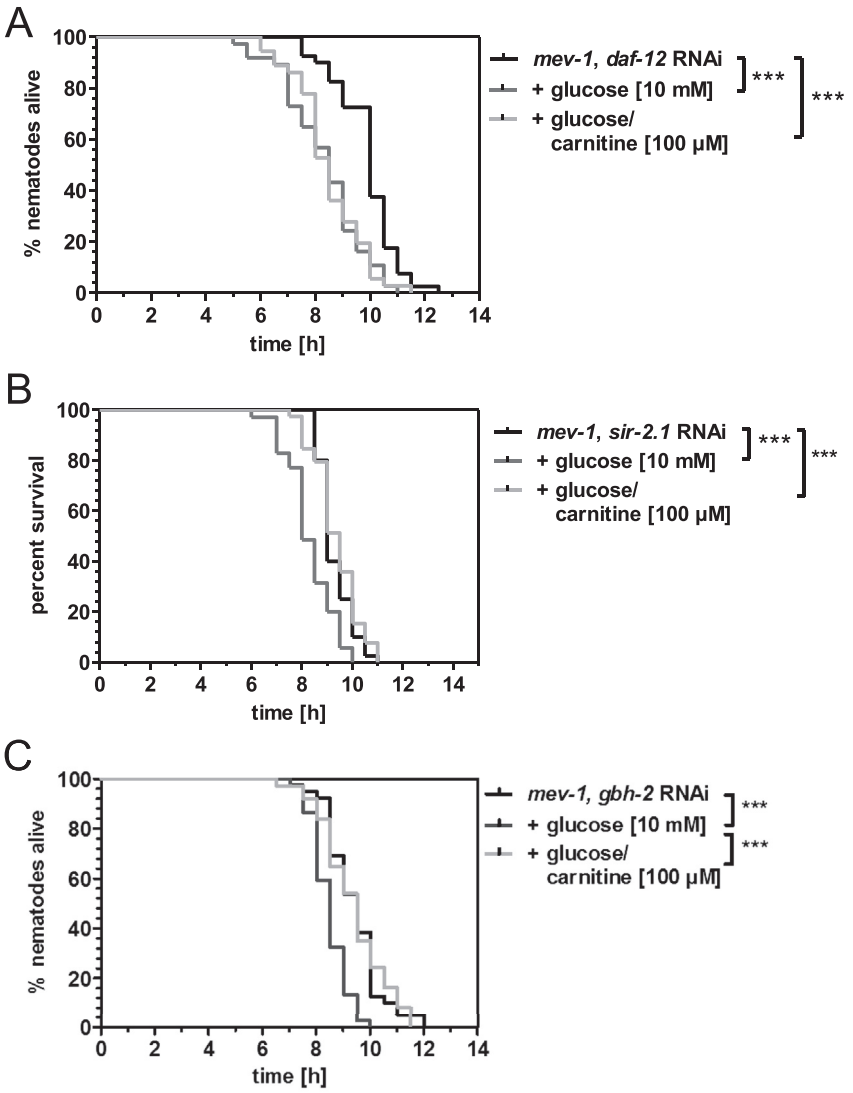

Fig. 2. Knockdown of *daf-12* prevents the survival rescue by carnitine. RNAi for *daf-12* as accomplished by feeding HT115 RNAi bacteria from the L1 stage on, prevented the extension of survival by 100 µM carnitine in glucose treated *mev-1* nematodes (A). RNAi for *sir-2.1* did not display any effects on the survival extension caused by carnitine in glucose exposed *mev-1* (B). Likewise did the knockdown of *gbh-2* not affect the effects of carnitine on survival extension (C). Vector controls did not display significant differences of survival compared to RNAi for *daf-12*, *sir-2.1* or *gbh-2*, respectively, and are not shown for reasons of clarity.

4. Discussion

L-carnitine is an essential part of the carnitine shuttle that is necessary to import fatty acids into mitochondria in order to enable their β-oxidation [27]. Moreover, by providing a pool for acetyl-CoA it decreases the acetyl-CoA/CoA ratio in the mitochondrial matrix and thereby releases the product inhibition of the pyruvate dehydrogenase complex [1,28]. This has been finally shown to enable an increased glucose oxidation which is able to explain the improved glucose homeoestasis and insulin-sensitivity observed in diabetic patients treated with carnitine [29].

In the model organism *C. elegans* used in the present study, carnitine applied at a concentration of 100 µM was able to completely prevent the reduction of survival caused by the addition of 10 mM glucose. Glucose concentrations were set in order to achieve levels in the nematodes which represent diabetes relevant concentrations of about 8 mM [30], whereas carnitine concentrations of 100 µM represent human plasma levels after exogenous carnitine administration [31]. Since we have found in our previous study that the polyphenol quercetin protects *mev-1* nematodes to the same extent as carnitine depending on *sir-2.1* and the nuclear hormone receptor *daf-12*, we tested the influence of those factors also in the present study. Interestingly, the survival extending effects of carnitine were completely independent of the sirtuin activity. *Daf-12*, however, proved essential in order to allow carnitine to exert its stress-resistance enhancing effects. It has been described, that DAF-12 interacts with the *C. elegans* forkhead transcription factor, DAF-16, paralleling the interaction between PPAR-γ and Foxo1 in mammalians [32]. However, so far there is only very little information about the functional convergence of the nematode Foxo-1 homolog DAF-16 and PPAR-γ homolog DAF-12 and although the mammalian factors antagonize each other, our previous study showed that the effects of quercetin were dependent on *daf-12* but independent of *daf-16* [17].

Interestingly, activity of the proteasome, which was increased due to exposure to quercetin [18], was not only inhibited by glucose, as described also in diabetic mice [33], but even more by carnitine. An inhibition of the proteasome has been described for

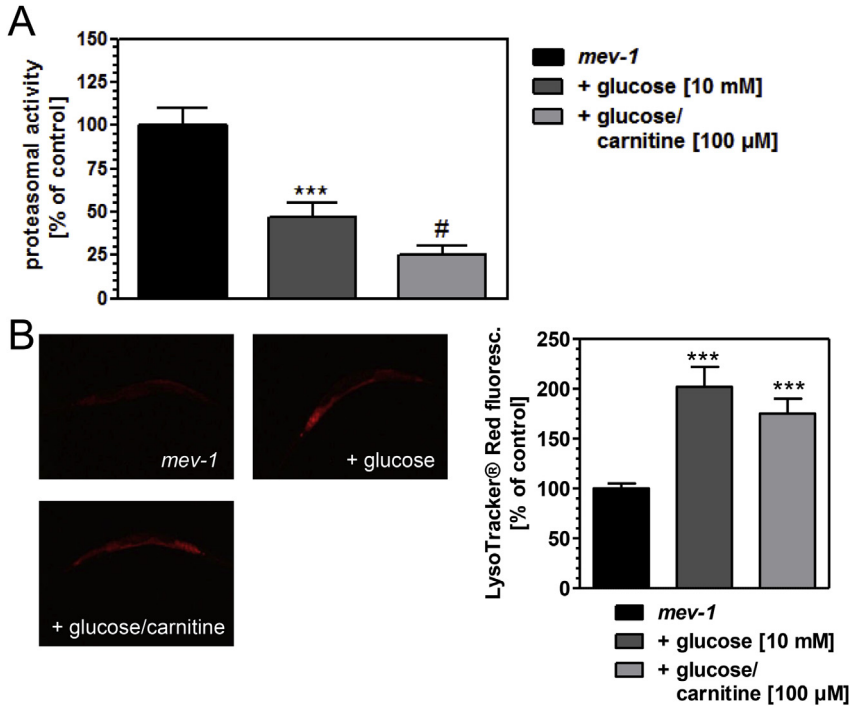

Fig. 3. Protein degradation pathways are not involved in the survival extension caused by carnitine. Chymotrypsin-like proteasomal activity was estimated according to the cleavage of Suc-LLVY-AMC in the absence and presence of the proteasome inhibitor MG132 as described in the Methods section. Proteasomal activity was significantly reduced by glucose and a further reduction was observed after additional application of carnitine (A). In contrast, the extent of lysosomes, as determined by LysoTracker® Red fluorescence, was increased by glucose whereas the simultaneous application of carnitine had no further effect (B). ***$p < 0.001$ versus the control, #$p < 0.05$ versus glucose treated *mev-1*.

carnitine also in the skeletal muscle of rats [34] and let us therefore suggest that carnitine increases the stress-resistance independent of the proteasomal activity. A second important pathway for protein degradation is autophagy which finally results in the formation of lysosomes in order to eliminate aggregated proteins, damaged organelles, and intracellular pathogens [35]. Glucose application markedly increased the formation of lysosomes, probably in order to degrade increasingly accumulating and aggregating non functional proteins. The triggering of this pathway, however, seems not to be beneficial for glucose treated nematodes, since blocking it prevents glucose toxicity by enhancing simultaneously proteasomal degradation [16]. Independent of the significance of autophagy for glucose mediated survival reduction, this pathway was found not to be affected by carnitine here.

Although the exact mechanisms through which carnitine affects stress-resistance and thereby survival in *C. elegans* have to be unraveled, it is suggested that DAF-12 and PPAR-γ share functional homologies, such as the regulation of various genes involved in glucose homeostasis [36]. The fact that decreased binding of PPAR-γ to the *cis*-acting PPAR-response element of several target genes with functions in glucose metabolism has been demonstrated fits into such a concept [37]. Finally, likewise to PPAR-γ, DAF-12 could stimulate the biosynthesis of carnitine by increasing the expression of γ-butyrobetaine dioxygenase, the key enzyme in carnitine biosynthesis pathway [38]. Those effects, however were not relevant for the survival extension by exogenous carnitine in the present study since it remained unaltered after the knockdown of γ-butyrobetaine dioxygenase. Alternatively, the increased expression level of OCTN2, the transporter mediating cellular carnitine uptake, could explain the dependence of the carnitine effects on the PPAR-γ homologue DAF-12 [39]. Our studies show that carnitine supplementation is able to enhance the resistance against heat-stress and thereby to increase the survival of *mev-1* nematodes. The nuclear hormone receptor DAF-12 was identified as a key factor mediating those effects.

Conflict of interest

None.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.101.

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