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Caenorhabditis elegans as a Model To Study the Effectiveness and Metabolic Targets of Dietary Supplements Used for Obesity Treatment: The Specific Case of a Conjugated Linoleic Acid Mixture (Tonalin)

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ABSTRACT: The antiobesity effect of conjugated linoleic acid (CLA) has previously been described in different animal models. The aim of the present study was to investigate the effect of a commercial mixture (Tonalin) on *Caenorhabditis elegans* to assess their potential use for functional ingredient screenings. Body-fat reduction with Tonalin was demonstrated in wild-type strain N2. The 1 μg/mL dose was the most effective, either alone or added to a food matrix, and also significantly decreased triglyceride content in nematodes fed on the CLA mixture. Furthermore, the antiobesity effect was related to the CLA isomer *trans*-10, *cis*-12. Finally, the transcriptional study showed *C. elegans* fed with Tonalin (1 μg/mL) underwent an upregulation of energy metabolism, reproduction, protein metabolism and oxidative stress processes. In conclusion, the results presented here clearly correlate well with other animal studies, demonstrating the value of *C. elegans* as a useful model to evaluate antiobesity compounds/ingredients.

KEYWORDS: *obesity, conjugated linoleic acid (CLA), Caenorhabditis elegans, transcriptional response*

INTRODUCTION

Obesity is a multifactorial disorder defined by a body-mass index (BMI) of ≥30 kg/m^2 and is considered a risk factor for diseases such as type II diabetes, cardiovascular disease, hypertension and certain types of cancer. It occurs when energy intake exceeds energy expenditure and is influenced by factors such as diet, developmental stage, physical activity and genetics.[1,2] Obesity (and overweight) is becoming one of the greatest threats to global health this millennium, affecting more than 50% of the adult population between 35 and 65 years. The prevalence of obesity has increased in recent years,[2,3] leading to an increase in associated disorders and healthcare costs. To date, obesity treatments are based on interventional strategies such as diet, exercise or drugs, although dietary supplements are also widespread as they improve successful weight management.[4]

An important group of compounds used as dietary supplements comprises conjugated linoleic acid (CLA), a mixture of conjugated octadecadienoic acid isomers derived from linoleic acid, naturally present in beef and dairy products.[5] Isomers with well-known biological activity are *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA. The beneficial properties of CLA are widely reported and include antiobesity, anticarcinogenic, antidiabetic and immune stimulation effects.[6−8] Concerning obesity, several animal and human studies have shown that CLA reduces body-fat mass and change body composition.[9−12] Furthermore, comparative studies have shown the isomer *trans*-10, *cis*-12 to be the most effective in lipid reduction of adipocytes.[13−15] Thus, these studies provide strong evidence of the capacity of CLA to reduce adiposity in animals. However, the effects of CLA on body weight and body composition in humans are controversial and studies are contradictory, depending on the isomers and/or doses used, treatment period and study population.[16]

Notwithstanding, CLA food supplementation can provide health benefits, as consuming these products could compensate for the low CLA intake in current diets. To date, there are few studies showing the effects of CLA added to food, and these are human intervention studies exclusively based on dairy product supplementation. Accordingly, significant fat-mass reduction after intake of CLA-supplemented milk has been reported in overweight human subjects but not in obese subjects.[17] Furthermore, abdominal body-fat reduction in overweight or obese children fed CLA in chocolate milk has been confirmed.[18] Conversely, other studies do not show differences in body composition after consumption of CLA-supplemented yogurt.[19,20] Therefore, further evaluation of the effectiveness of CLA-supplemented food matrixes on body-fat reduction is of great interest.

In the present study, we have used *Caenorhabditis elegans* to examine the potential antiobesity effect of a commercial mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers, Tonalin (Arkopharma, Carros, France). *C. elegans* is considered an excellent model to study obesity. Recent research has provided insight about the nematode's genes involved in the basic homeostatic pathways that regulate energy balance and fat storage, and many of these genes have human orthologues.[21−23] Moreover, their short lifespan (21 days), together with body transparency and ease of culture in agar dishes, encourages their

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increasingly common use to evaluate potential obesity therapeutics.[24−26] The objective of this study was to validate the effect of CLA on body fat reduction in our model organism using the lipid-staining Red Nile dye and triglyceride measurement. CLA was administered either alone or added to a food matrix (skimmed milk and orange juice). Furthermore, as previously shown in other evaluation models,[27,28,6,7] our microarray analysis also showed the effect of Tonalin on energy expenditure and oxidative stress response in the nematode. Our results clearly demonstrate the potential use of *C. elegans* as a model organism for faster and less expensive analysis of dietary supplements or functional food ingredients for obesity treatment or weight control.

MATERIALS AND METHODS

C. elegans Strain and Treatments. *C. elegans* strain N2, Bristol (wild-type), was obtained from the *Caenorhabditis* Genetics Centre (CGC) at the University of Minnesota (USA) and maintained at 20 °C on nematode growth medium (NGM). *Escherichia coli* OP50 strain was used as normal nematode diet and was also provided by the CGC.

Worms were grown on NGM as control diet or NGM supplemented with different doses (0.1, 0.5, 1, 10, 50 and 100 μg/mL) of a commercial mixture of conjugated linoleic acid (CLA), named Tonalin (Arkopharma, Carros, France). Nutritional composition of Tonalin is described in Table 1. The Tonalin-containing medium was prepared by mixing the appropriate amount of a stock solution to the medium to obtain the corresponding concentrations. All NGM plates contained *Escherichia coli* OP50.

Table 1. Nutritional Composition of the Commercial Mixture of CLA Tonalin (Arkopharma, Carros, France)

	g/100 g of product
proteins	19.6
carbohydrates	9.1
total lipids[a]	71.3
CLA	24.9
not CLA	46.4

[a]Containing 39% *cis*-9, *trans*-11; 41% *trans*-10, *cis*-12; 5% *cis*-9, *cis*-11 and *cis*-10, *cis*-12; 0.5% linoleic acid; 5.5% oleic acid and 4% other not identified fatty acids.[34]

In addition, nematodes were also fed the main isomers contained in the commercial product Tonalin (39%of *cis*-9, *trans*-11; 41% *trans*-10, *cis*-12, refs 17 and 34). Regarding pure isomers, *cis*-9, *trans*-11 and *trans*-10, *cis*-12, stock solutions were prepared in ethanol and added to the NGM medium at 1, 10, 50 and 100 μg/mL final concentration.

Furthermore, the bioactivity of Tonalin on *C. elegans* body-fat reduction in the presence of skimmed milk or orange juice was evaluated. The experiments were performed on NG plates supplemented with commercial skimmed milk or orange juice (50 μL/plate). Tonalin was added to skimmed milk or orange juice to obtain 1 or 100 μg/mL of final concentration in the plates. Nematodes were fed under the different conditions until reaching young adult stage, and then total lipid content was determined by Red Nile staining.

Red Nile Staining. The effects of Tonalin and the main isomers of CLA (*cis*-9, *trans*-11 and *trans*-10, *cis*-12) on body-fat reduction in *C. elegans* N2 were studied by measuring fluorescence of stained worms. Populations of age-synchronized worms were obtained by isolating eggs from gravid adults and hatching the eggs overnight in NGM plates (as control media), NGM plates with 6 μg/mL of Orlistat (Sigma-Aldrich, Madrid, Spain) used as positive control and NGM plates supplemented with the different doses of Tonalin or the aforementioned pure isomers contained in CLA.

Nile Red (9-diethylamino-5*H*-benzo[α]phenoxazin-5-one, Sigma, St. Louis, MO, USA) was used as dye to monitor lipid storage in live worms. The dye was added on the top of the NGM agar plates, preseeded with *Escherichia coli* OP50, to a final concentration of 0.05 μg/mL. Worms were incubated at 20 °C for 3 days until young adult stage. After this incubation period, nematode samples were placed in M9 buffer and fluorescence was measured in an FP-6200 system (JASCO Analytical Instruments, Easton, MD, USA) using λ excitation 480 nm and λ emission 571 nm. A total of 90 worms per condition were analyzed. Experiments were carried out in triplicate.

Triglyceride Assay. Nematode triglyceride content was measured using Triglyceride Quantification Kit (Biovision, Mountain View, CA). Age-synchronized nematodes were treated with Tonalin (1 or 100 μg/mL), *cis*-9, *trans*-11 (10 μg/mL) or *trans*-10, *cis*-12 (1 μg/mL) until young adult stage. Worms were then collected and washed with PBS buffer. After worm settling, supernatant was removed and 400 μL of the triglyceride assay buffer was added to worm pellet. Worms were sonicated with a digital sonifier (Branson Ultrasonics Corporation, Danbury, CT, USA) using 4 pulses for 30 s at 10% power. Total protein content was estimated by BCA. Samples were slowly heated twice at 90 °C for 5 min in a thermomixer (ThermoFisher) to solubilize all triglycerides in the solution. After brief centrifugation, samples were used for the triglyceride assay (50 μL/well) following the manufacturer's instructions. Five different biological replicates were included for each condition in three independent experiments.

Oxidative Stress Assays. After acute oxidative stress, survival rates were measured in nematodes fed with or without Tonalin (at 1 or 100 μg/mL of final concentration). Experiments were performed according to a previously published protocol.[29] Ascorbic acid (0.1 μg/mL, Sigma-Aldrich, St. Louis, MO, USA) was used as antioxidant positive control. Experiments were carried out in triplicate.

Microarray Analysis. Gene expression in *C. elegans* (young adults) cultured in Tonalin-supplemented NGM (1 μg/mL) was compared with nematodes grown in control conditions (NMG medium). Four biological replicates were examined per condition. Synchronized populations were obtained from embryos isolated from gravid adults in the different feeding conditions. Once the worm population reached the young adult stage, samples were collected with M9 buffer, washed three times and collected in eppendorf tubes for worm disruption by sonication. Total RNA was isolated with RNAasy Kit (Qiagen, Barcelona, Spain). RNA samples were processed for hybridization using the GeneChip *C. elegans* Genome Array (Affymetrix, UCIM, University of Valencia, Spain). Data were examined using bioinformatics.

Statistical Analysis. The significance of *C. elegans* body-fat reduction between control and treatment was analyzed by Student's *t* test (Stat Graphics software package). The significance in increased viability in treated worms after acute oxidative stress was also studied using this test.

Raw data from Affymetrix arrays were background corrected using RMA methodology.[30] Intensity signal was standardized across arrays via quantile-based normalization algorithm. Differential gene expression between control and treated conditions was assessed using limma moderated *t*-statistics. The *P* values obtained for each gene were adjusted with multiple testing *P* value correction procedures.[31] Finally, gene set analysis was performed for each comparison using logistic regression models.[32]

RESULTS

Tonalin Produces a Reduction of Red Nile Staining in _C. elegans_ N2 in a Dose-Dependent Manner. The results of measuring fluorescence of Red Nile stained synchronized nematode populations to determine Tonalin-related body-fat reduction are shown in Figure 1a. The figure shows the percentage of fluorescence obtained in nematodes cultured in control conditions and with different doses of Tonalin. The 1 μg/mL Tonalin concentration was the most effective on lipid reduction, with 36.4% fat reduction ($P < 0.001$). Tonalin at 0.5 μg/mL also produced a reduction in fluorescence (23.9%) ($P < 0.01$), while fluorescence reduction rates in nematodes fed with

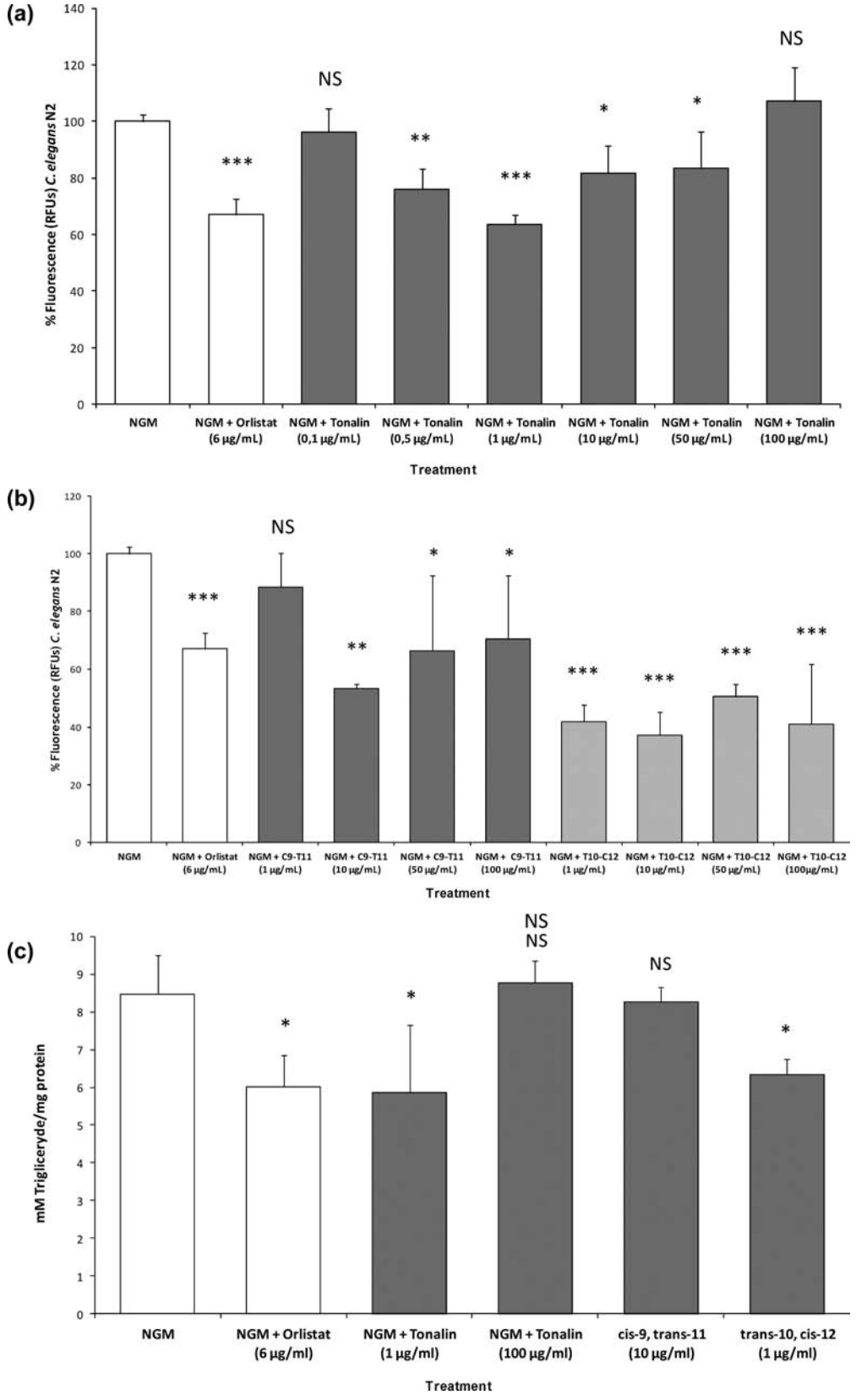

Figure 1. Quantification of fluorescence in Red Nile stained worms (wild-type strain N2). (a) Worms fed with different doses of a commercial CLA (Tonalin) (dark gray) or in control conditions (NGM or Orlistat) (white). (b) Worms fed with the main CLA isomers *c*9-*t*11 (dark gray) and *t*10-*c*12 (light gray). NGM was used as reference feeding condition. Percentage of fluorescence is the mean of three independent experiments. (c) Triglyceride content measured in *C. elegans* N2 fed with most effective doses of Tonalin, *c*9-*t*11 and *t*10-*c*12 (dark gray). ***Significant *P* value ≤0.001. **Significant *P* value ≤0.01. *Significant *P* value ≤0.05. NS: Not significant.

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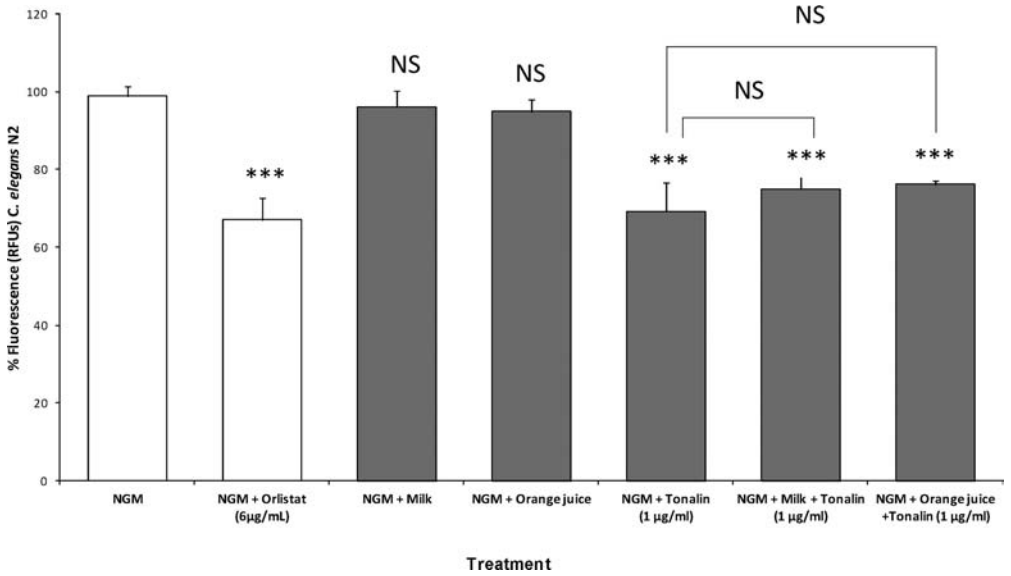

Figure 2. Supplementation of skimmed milk or orange juice with Tonalin (1 μg/mL) produces body fat reduction on *C. elegans*. Milk and orange juice were added at a volume of 50 μL/plate. NGM was used as control feeding condition and Orlistat (6 μg/mL) as positive control. Test conditions: dark gray. Control conditions: white. ***Significant P value $\leq$0.001. NS: Not significant.

10 and 50 μg/mL of Tonalin were 18.35 and 16.57% ($P < 0.05$), respectively. Conversely, no significant decrease in fluorescence was observed using 0.1 μg/mL or 100 μg/mL of Tonalin. These results indicate that, in *C. elegans,* Tonalin-related fat reduction is dose-dependent, the most effective concentration being that of 1 μg/mL.

Tonalin comprises two major isomers, *cis*-9, *trans*-11 and *trans*-10, *cis*-12 (50% *cis*-9, *trans*-11 and 41% *trans*-10, *cis*-12), with other CLA isomers at considerably lower levels. Although *cis*-9, *trans*-11 is the main CLA isomer in natural food,[17] the isomer *trans*-10, *cis*-12 is reported to affect energy metabolism and body-fat deposition and composition in mice.[13] Other studies in murine 3T3-L1 adipocytes have shown smaller lipid droplets in differentiated cells treated with *trans*-10, *cis*-12 CLA[14] as well as a higher influence on lipid metabolism, energy expenditure and fatty acid oxidation in 3T3-L1 adipocytes.[15,7] In humans results are controversial and studies have not shown either *cis*-9, *trans*-11 or *trans*-10, *cis*-12 CLA isomer to significantly affect body weight.[16] Only one study carried out with human adipose tissue showed a stronger effect on gene expression after treatment with *trans*-10, *cis*-12 compared with *cis*-9, *trans*-11 CLA isomer.[33]

To study the specific effect of each isomer contained in Tonalin, we evaluated fat reduction in nematodes treated with different doses of both CLA isomers by Red Nile staining. Results (Figure 1b) show that *cis*-9, *trans*-11 and *trans*-10, *cis*-12 were able to reduce the body fat in *C. elegans* N2 strain. However, the effect was particularly notable for isomer *trans*-10, *cis*-12, as a highly significant reduction was observed for all doses assayed ($P \leq 0.001$). Percentage of fluorescence reduction with *trans*-10, *cis*-12 was between 49.6 and 62.7%, indicating this isomer was more effective than Tonalin. This could be explained by the complex composition of the commercial product, which also contains low quantities of other lipids, proteins and carbohydrates that could interfere in the specific effect of CLA isomers included in the product. Differences in fluorescence measurements corresponding to the different doses of *trans*-10, *cis*-12 CLA were very low. In the case of isomer *cis*-9, *trans*-11, only concentrations of 10 ($P \leq 0.01$), 50 and 100 μg/mL ($P \leq 0.05$) produced a significant reduction in nematode fluorescence, showing percentages between 43.3 and 29.6%. No significant reduction was determined with 1 μg/mL of *cis*-9, *trans*-11.

Triglyceride Quantification Supports the Antiobesity Effect of Tonalin in *C. elegans*. Furthermore, the antiobesity effect of Tonalin and both CLA isomers was validated by triglyceride quantification in nematodes treated with the most effective doses (1 μg/mL of Tonalin, 10 μg/mL of *cis*-9, *trans*-11 isomer or 1 μg/mL of *trans*-10, *cis*-12 isomer). A Tonalin concentration of 100 μg/mL was also tested as negative control in the triglyceride assay, having been shown ineffective in the fluorescence test. Figure 1c shows that triglyceride content of strain N2 under control conditions (NGM) was 8.46 mM/mg protein, while for 1 μg/mL Tonalin the triglyceride concentration in this strain was lower (5.85 mM/mg protein). This was consistent with the effect observed by lipid droplet staining. Again, the dose of 100 μg/mL of Tonalin was ineffective in our model, as no triglyceride reduction was found under this condition. Further analysis showed a significant effect of isomer *trans*-10, *cis*-12, which produced a reduction in triglycerides from 8.46 (control conditions) to 6.34 mM/mg protein in treated nematodes (Figure 1c). Meanwhile, no significant triglyceride reduction was observed for *cis*-9, *trans*-11 isomer. These results are in accordance with previous literature revealing the specific effect of isomer *trans*-10, *cis*-12 on body-fat reduction.[5,33,34]

Tonalin-Supplemented Food Is Effective on Body Fat Reduction in the Nematode. Once the effect of Tonalin on nematode fat reduction had been demonstrated, the effects of Tonalin-supplemented food were studied. Skimmed milk and orange juice were selected as food matrix, and added to each plate (50 μL of final volume) subsequent to a dose–response assay (data not shown). Tonalin was supplemented at 1 μg/mL, as this was the most effective dose on lipid reduction in the nematode. Figure 2 shows that milk or orange juice supplemented with 1 μg/mL of Tonalin reduces body fat in *C. elegans* ($P \leq 0.001$). Moreover, results showed no significant effects of the vehicle (skimmed milk or orange juice) on body-

fat reduction of nematodes as no significant differences were determined between Tonalin (1 μg/mL) and the Tonalin mixed with skimmed milk or orange juice.

Energy Expenditure and Oxidative Stress Response Are Metabolic Targets of Tonalin in *C. elegans*. In the next step, the transcriptional response in *C. elegans* was studied using microarray experiments to analyze the mechanism underlying the effects of Tonalin. Worms were exposed to 1 μg/mL of the product (most effective dose), and RNA from animals was used for hybridization to Affymetrix *C. elegans* arrays. Four biological replicates were analyzed to determine gene expression patterns from *C. elegans* N2 fed NGM with or without Tonalin. Results showed gene pattern expression remained unchanged in nematodes fed with Tonalin at this dose compared with the control. However, ontology enrichment analysis showed a total of 38 upregulated biological processes in worms fed with 1 μg/mL of Tonalin (Table 2). Among these biological processes, the first set of genes were involved in the generation of metabolites and energy (such as oxidative phosphorylation, ATP metabolic process, ATP synthesis-coupled proton transport and organic acid metabolic process). This is in agreement with a previous study,[7] which showed that CLA isomers can regulate mitochondrial-membrane transporter expression in 3T3-L1 cells in order to modulate metabolic efficiency and increase energy expenditure. Furthermore, different studies in animal models support this, as they show the effect of CLA on the increase in energy expenditure through body mass reduction and thermogenesis via ATP synthesis.[6] Other biological processes affected in nematodes fed with Tonalin were related to sexual reproduction (gamete generation, fertilization). It has previously been shown that the reproductive status of simple organisms like *C. elegans* is modulated by nutrient availability, indicating there is a metabolic regulation of fertility.[35] Moreover, the insulin-like signaling pathway plays an important role in the regulation of fertility, metabolism and longevity in the nematode[36] and this mechanism is conserved in mammals.[35] Our results indicate that Tonalin produces an increase in metabolic processes related to nematode reproduction, leading to a reduction in its lipid content. Taking into account that obesity has been associated with infertile conditions,[37] our results would indicate that reproduction and energy homeostasis are coordinated processes that modulate lipid content in the nematode.

Other biological processes upregulated in the nematodes after Tonalin supplementation were translation (ribonucleotide biosynthetic process), cell localization and organization (such as nuclear migration or nuclear envelope organization and establishment of nucleus localization), cellular response to stimulus and response to oxidative stress and protein folding. These results might indicate the important role of Tonalin in enhancing protein metabolism and oxidative stress response. Regarding the former, there is no previous transcriptomic research reporting the effect of CLA on protein metabolism. However, our results might indicate their role in slowing down aging through protein homeostasis activation. On the other hand, the observed transcriptomic response to oxidative stress produced by Tonalin was confirmed by survival analysis in nematodes treated with Tonalin and subjected to acute oxidative stress. Results indicate a marked antioxidant effect of Tonalin in a dose dependent manner (Figure 3), in agreement with other authors reporting the anti-inflammatory activity of CLA and their potential use for sarcopenia prevention.[27,28]

Table 2. List of Significantly ($P \leq 0.05$) Enriched Biological Processes Observed in *C. elegans* N2 after Administration of 1 μg/mL of Tonalin vs Control Feeding Conditions (NGM)[a]

upregulated GO biological processes			
ID (GO)[b]	name	size	*P* value
GO:0006091	generation of precursor metabolites and energy	122	0.0009
GO:0019953	sexual reproduction	416	0.0009
GO:0006412	translation	339	0.0024
GO:0007338	single fertilization	79	0.0100
GO:0009566	fertilization	85	0.0100
GO:0006119	oxidative phosphorylation	62	0.0100
GO:0051656	establishment of organelle localization	148	0.0100
GO:0051640	organelle localization	153	0.0100
GO:0007097	nuclear migration	83	0.0213
GO:0035046	pronuclear migration	75	0.0259
GO:0006818	hydrogen transport	53	0.0259
GO:0046034	ATP metabolic process	94	0.0259
GO:0051647	nucleus localization	84	0.0259
GO:0040023	establishment of nucleus localization	84	0.0259
GO:0042180	cellular ketone metabolic process	292	0.0271
GO:0006520	cellular amino acid metabolic process	181	0.0293
GO:0015986	ATP synthesis coupled proton transport	47	0.0295
GO:0015985	energy coupled proton transport	47	0.0295
GO:0009260	ribonucleotide biosynthetic process	110	0.0307
GO:0006812	cation transport	375	0.0323
GO:0006998	nuclear envelope organization	10	0.0337
GO:0007276	gamete generation	335	0.0341
GO:0019752	carboxylic acid metabolic process	284	0.0342
GO:0006082	organic acid metabolic process	284	0.0342
GO:0043436	oxoacid metabolic process	284	0.0342
GO:0044271	cellular nitrogen compound biosynthetic process	260	0.0356
GO:0009152	purine ribonucleotide biosynthetic process	106	0.0365
GO:0051789	response to protein stimulus	28	0.0365
GO:0044283	small molecule biosynthetic process	289	0.0365
GO:0015992	proton transport	51	0.0365
GO:0071216	cellular response to biotic stimulus	27	0.0365
GO:0071445	cellular response to protein stimulus	27	0.0365
GO:0006979	response to oxidative stress	56	0.0381
GO:0034404	nucleobase, nucleoside and nucleotide biosynthetic process	186	0.0457
GO:0034654	nucleobase, nucleoside, nucleotide and nucleic acid biosynthetic process	186	0.0457
GO:0034220	ion transmembrane transport	56	0.0466
GO:0006457	protein folding	101	0.0466
GO:0006754	ATP biosynthetic process	91	0.0466

[a]Size (number of genes composing the group) and *P* value are indicated. [b]GO, Gene Ontology.

Furthermore, analysis showed a group of downregulated processes in nematodes fed with Tonalin (Table 3). These 13 processes were related to dosage compensation, nucleosome assembly, chitin catabolic process and dendrite development.

Finally, functional gene set analysis was performed using the KEGG pathway database to search for enriched pathways in nematodes fed with 1 μg/mL Tonalin (Table 4). Results showed ten upregulated pathways with statistical significance ($P \leq 0.05$) in treated nematodes. These pathways are involved in

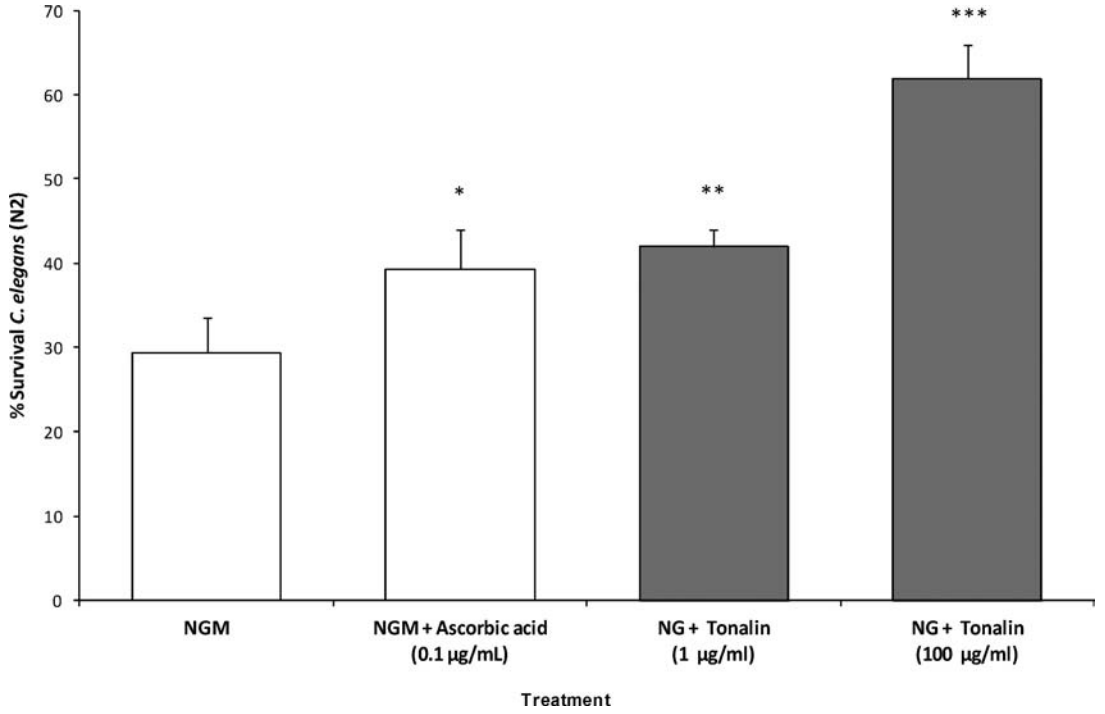

Figure 3. Antioxidant activity of Tonalin in *C. elegans* N2. Percentage of worm survival was measured after an acute oxidative stress with H_2O_2 (2 mM). Tonalin was added to the NGM plates at 1 or 100 μg/mL of final concentration. Ascorbic acid (0.1 μg/mL) was used as positive control. Test conditions: dark gray. Control conditions: white. ***Significant P value $\leq$ 0.001. **Significant P value $\leq$ 0.01. *Significant P value $\leq$ 0.05.

Table 3. List of Significantly ($P \leq 0.05$) Downregulated Biological Processes Observed in *C. elegans* N2 after Administration of 1 μg/mL of Tonalin vs Control Fed Conditions (NGM)[a]

downregulated GO biological processes			
ID (GO)[b]	name	size	P value
GO:0042464	dosage compensation, by hypoactivation of X chromosome	17	0.0055
GO:0007549	dosage compensation	19	0.0100
GO:0006325	chromatin organization	110	0.0100
GO:0006323	DNA packaging	60	0.0139
GO:0031497	chromatin assembly	50	0.0259
GO:0034728	nucleosome organization	49	0.0259
GO:0006334	nucleosome assembly	49	0.0259
GO:0065004	protein−DNA complex assembly	56	0.0271
GO:0016358	dendrite development	10	0.0424
GO:0006030	chitin metabolic process	49	0.0342
GO:0006032	chitin catabolic process	35	0.0323
GO:0006333	chromatin assembly or disassembly	78	0.0307
GO:0006333	chromatin assembly or disassembly	78	0.0307

[a]Size (number of genes composing the group) and P value are indicated. [b]GO, Gene Ontology.

Table 4. List of Significantly ($P \leq 0.05$) Upregulated Metabolic Pathways Observed in *C. elegans* N2 after Administration of 1 μg/mL of Tonalin vs Control Fed Conditions (NGM)[a]

upregulated KEGG pathways			
ID (KEGG)[b]	name	size	P value
190	oxidative phosphorylation	127	0.0025
3010	ribosome	115	0.0032
620	pyruvate metabolism	26	0.0035
20	citrate cycle (TCA cycle)	42	0.0142
230	purine metabolism	114	0.0142
3060	protein export	24	0.0185
260	glycine, serine and threonine metabolism	23	0.0341
510	*N*-glycan biosynthesis	40	0.0341
290	valine, leucine and isoleucine biosynthesis	14	0.0419
3018	RNA degradation	57	0.0470

[a]Size (number of genes composing the group) and P value are indicated. [b]KEGG, Kyoto Encyclopedia of Genes and Genomes.

energy metabolism (oxidative phosphorylation), carbohydrate metabolism (pyruvate metabolism, citrate cycle) and protein metabolism (ribosome, protein export, amino acid metabolism), being consistent with the observed upregulated biological processes. No downregulated pathways were determined in Tonalin-treated nematodes.

DISCUSSION

The beneficial properties of CLA, including anticarcinogenic, antidiabetogenic and immune modulating effects, have been widely reported in different biological systems.[38,39,8] Concerning antiobesity properties, a high number of studies in different animal models have shown the effect of CLA on body weight and body lipid composition.[6] Thus, several reports have shown the ability of CLA to reduce adiposity and increase lean mass in mice and Zucker rats.[9,5,11] Regarding experiments in humans, there are reports with contradictory results. Some studies have provided evidence for the effects of conjugated linoleic acid on body weight or body-fat mass reduction. Thus, mixtures of *trans*-10, *cis*-12 and *cis*-9, *trans*-11 CLA were shown to produce a significant reduction in body-fat mass, but not in body weight reduction.[40] Other studies have reported significant body weight reduction in human subjects administered CLA, indicating levels of reduction between 1 and 2%.[41,42]

Conversely, there are many studies that have not shown any significant antiobesity effects of CLA in human trials.[8] In the present study we demonstrate the antiobesity effect of CLA in the *Caenorhabditis elegans* model using a commercial mixture of isomers *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA (Tonalin). In addition, the observed effect was dose dependent, the most effective dose being 1 μg/mL. Furthermore, we demonstrate that the main antiobesity properties of Tonalin can be attributed to *trans*-10, *cis*-12 CLA isomer, as it produced a marked lipid reduction in nematodes. This is consistent with previous studies indicating the *trans*-10, *cis*-12 isomer as the active isomer with regard to antiobesity in different experimental models such as cell cultures[7] and animals.[13,16,43] Unfortunately, the antiobesity effects of *trans*-10, *cis*-12 CLA isomer in humans remain unclear due to the presence of other intrinsic experimental parameters such as the dose used, study length or exercise.[10,44] Herein, the effectiveness of Tonalin added to a food matrix has been demonstrated. Our results suggest that skimmed milk supplemented with Tonalin has a positive effect on *C. elegans* body-fat reduction, in accordance with previous reports which showed a significant reduction in fat mass in overweight persons.[17,18]

Furthermore, we have used the *C. elegans* model to elucidate the molecular mechanism triggered by Tonalin at the effective dose of 1 μg/mL. Surprisingly, no specific genes with differential expression were determined, probably due to the low dose used. This hypothesis is based on the fact that higher doses of Tonalin (100 μg/mL) produced a stronger gene expression response in the nematode (data not shown). Further microarray analysis demonstrated that administering nematodes 1 μg/mL of Tonalin induced an upregulation in metabolic processes related to energy expenditure, carbohydrate metabolism, sexual reproduction, protein metabolism and oxidative stress response. The effect of CLA on energy expenditure and fatty acid oxidation in 3T3-L1 cells has previously been reported.[7] Both isomers, *cis*-9, *trans*-11 and *trans*-10, *cis*-12, act by modulating the gene expression of mitochondrial inner-membrane transporters (UCPs) and the $PPAR_{\alpha}$ gene. In the present study, we demonstrate the effect of Tonalin (50% *cis*-9, *trans*-11 and 41% *trans*-10, *cis*-12) on the upregulation of oxidative phosphorylation in *C. elegans*. An upregulation of the citrate cycle was also observed in nematodes fed with 1 μg/mL of Tonalin, suggesting that CLA enhances the oxidation of carbohydrates and fatty acids, and then the energy (NADH) released in the CTA cycle is used in oxidative phosphorylation for ATP synthesis. This is consistent with the phenotype (reduced fat) observed in nematodes fed with Tonalin, and is in agreement with previous studies showing a high-fat phenotype in nematodes with reduced expression of genes encoding fatty acid β-oxidation enzymes.[45] Moreover, energy homeostasis and reproduction have been described as coordinated processes in *C. elegans*,[35] being consistent with the observed effect of Tonalin on body-fat reduction and the upregulation of metabolic pathways related with reproduction and energy production.

In addition, our results also suggest that nematodes fed with Tonalin could increase acetyl CoA production, the main product of pyruvate metabolism, leading to an increase in ATP synthesis (through citrate cycle). Taking into account that the pyruvate metabolism is a central pathway which interconnects different related metabolic pathways, we hypothesize that the observed upregulation of the pyruvate metabolism is related to the upregulation of amino acid and protein metabolism observed in the treated nematodes. To date, there are no transcriptomic studies showing any effect of CLA on upregulation of protein metabolism/synthesis in any animal model. Our results clearly indicate an upregulation of protein folding, export and amino acid biosynthesis in nematodes fed on Tonalin, suggesting a possible role of CLA in nematode proteostasis. This fact, together with our results showing the antioxidant effect of the commercial mixture of CLA (both from transcriptomic and phenotypic analysis), demonstrates the potential of Tonalin to help prevent aging-related diseases. This is consistent with previous studies showing free radical scavenging properties of CLA.[46] Furthermore, the ability of CLA to control oxidative status in mice through biochemical analysis[27] and its potential use for prevention of sarcopenia in this animal model have been described.[28] Regarding human trials, although recent studies have indicated only a slight effect of CLA intake on oxidative stress biochemical markers,[47] further research in humans must be performed to shed light on the molecular mechanism controlling the oxidative stress protection provided by CLA.

In summary, the present study has shown the effectiveness of a commercial mixture of CLA, Tonalin, on body-fat reduction in the nematode *C. elegans*. This effect is dose-dependent and can mainly be attributed to the activity of isomer *trans*-10, *cis*-12, which also remains effective when added to a matrix, like skimmed milk or orange juice. Moreover, the molecular mechanism triggered in the nematodes fed with Tonalin has been identified and described, demonstrating how the CLA mixture affects its energy metabolism, reproduction, protein metabolism and oxidative stress response. Furthermore, the results presented here clearly correlate well with the findings of other studies on CLA performed in different animal models. Therefore, *C. elegans* has proven to be a powerful in vivo model to study functional food ingredients affecting obesity. In the case of human trials, there is also some evidence of the effect of CLA on body-fat reduction[40] and on the modulation of gene expression affecting fatty acid transport and metabolism.[33] However, further human trials are needed to gain insight into the molecular pathways affected by CLA in order to establish a good correlation between animal models and humans.

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Notes

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