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Trans-10, cis-12 Conjugated Linoleic Acid Induces Lipid Degradation and Attenuates Fatty Acid Desaturation in Yeast Saccharomyces cerevisiae

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Abstract

Yeast has been successfully utilized as a model organism to study the antilipogenic mechanism of the trans-10, cis-12 isomer of conjugated linoleic acid (CLA). The isomer lowered triacylglycerol content of yeast if supplemented in the cultivation medium. Also monounsaturated to saturated fatty acid ratio in yeast lipids was reduced in the CLA treated cells. In this study, the metabolic conditions in terms of lipid reduction were examined to clarify the antilipogenic mechanism of CLA in yeast. Triacylglycerol accumulation and degradation were examined and desaturation of trans-vaccenic acid to cis-9, trans-11 CLA was measured in the trans-10, cis-12 CLA treated cells. The study confirmed that triacylglycerol accumulation was not prevented in the trans-10, cis-12 CLA treated cells evidenced by the intensive co-accumulation of oleic acid. In the trans-10, cis-12 CLA treated culture the triacylglycerol content started to decrease and cellular biomass increase in the stationary phase after the cells had shifted their metabolism from fermentation to respiratory metabolism. This indicated that yeast used the liberated fatty acids for β-oxidation. The lowered capacity to desaturate the trans-vaccenic acid to cis-9, trans-11 CLA pointed out that also desaturase inhibition hasan important role in the antilipogenic mechanism of the trans-10, cis-12 CLA in yeast.

Keywords: Conjugated linoleic acid; Antilipogenic, Fatty acid desaturation; Fatty acid oxidation; Triacylglycerol hydrolysis; Yeast

Abbreviations: CLA: Conjugated Linoleic Acid; FA: Fatty Acid; MUFA: Monounsaturated FA; Ole1p: Δ9 desaturase; PPAR: Peroxisome Proliferator Activated Receptor; SFA: Saturated FA; TAG: Triacylglycerol; Tgl: Triacylglycerol Lipase

Introduction

Conjugated linoleic acids (CLA) are a group of linoleic acid isomers having double bonds in conjugated position, i.e. the double bonds are separated by one single bond. Two of the isomers: cis-9, trans-11 (c9,t11) and trans-10, cis-12 (t10, c12) CLA, have been shown to have biologically interesting properties. The c9,t11 CLA has been reported to have anticancer, anti-diabetic and anti-inflammatory properties whereas the t10, c12 CLA is known for its antilipogenic ability [1-3].

However, the exact mechanism of the lipid reducing action of t10, c12 CLA is unclear despite the intensive research with mammalian cells. Evidence for both inhibition of lipid accumulation [4-6] and increased lipid degradation [7-9] caused by t10, c12 CLA have been found. Clarification of the mechanism would be important in terms of recognizing potential detrimental effects of CLA. On the other hand, the knowledge of the connection between structure and action of CLA could be exploited when new antilipogenic substances are being developed.

Yeast Saccharomyces cerevisiae is a commonly used model organism in eukaryotic lipid metabolism studies. Lipid metabolic pathways share certain homology to higher eukaryotes and yeast might serve as an excellent tool to clarify the function of the t10, c12 CLA in the eukaryotic cell. If yeast S. cerevisiae was cultivated in the medium containing t10, c12 CLA, accumulation of triacylglycerols (TAG) was remarkably lower compared to control cultivations [10-12]. If yeast lipase activity was reduced by deleting the two major triacylglycerol lipases, Tgl3p and Tgl4p, TAG accumulated comparably to control irrespective of the presence of t10, c12 CLA, which indicated that TAG degradation was the reason for the lower TAG amount in the CLA treated wild type yeast [12].
One of the evidenced responses of mammals to t\textsubscript{10}, c\textsubscript{12} CLA administration is increased fatty acid (FA) oxidation. Higher release of CO\textsubscript{2} from C\textsubscript{16}-labeled oleic acid in preadipocytes in the presence of t\textsubscript{10}, c\textsubscript{12} CLA compared with the control cells reflecting increased FA oxidation was reported [8]. Studies of Zhai et al. [13] showed that both CLA isomers increased mRNA and protein levels of PPAR\textsubscript{\gamma}, a transcription factor controlling the genes of \(\beta\)-oxidation, but with t\textsubscript{10}, c\textsubscript{12} isomer the increment was multiple times higher, resulting in enhanced \(\beta\)-oxidation in 3T3-L1 preadipocytes. It was suggested that augmented FA oxidation by t\textsubscript{10}, c\textsubscript{12} CLA resulted in lower glucose uptake, which prevented the proper storage of the isomer in TAG, and the adipocytes were converted to cells that oxidize FA instead of storing them [9]. This assumption was based on the fact that disruption of FA oxidation restored lipid storage and glucose consumption. The authors also suggested that membrane incorporated t\textsubscript{10}, c\textsubscript{12} CLA might alter eg. the mitochondrial function due to specific stereochemistry of the isomer. In \textit{S. cerevisiae}, FA oxidation occurs in peroxisomes instead of mitochondria and \(\beta\)-oxidation is repressed in the glucose containing medium [14]. After glucose exhaustion, glycolytic metabolism is switched to gluconeogenesis and respiratory metabolism, which launches the usage of alternative carbon sources including FA oxidation [15]. The exogenously supplemented or internally released FA induce the expression of the enzymatic machinery for \(\beta\)-oxidation [16,17]. The expression of the enzymes for \(\beta\)-oxidation is controlled through the heterodimeric transcription factor Oaf1-Pip2, which binds to a specific sequence, the oleate response element (ORE) in its target genes [18]. Phosphorylation of Oaf1p (and possibly also Pip2p) is needed to activate the heterodimer in non-repressive conditions [19]. After recognition of the FA signal, the Oaf1-Pip2 undergoes a conformational change, which induces transcription of the peroxisomal genes.

Inhibition of FA desaturation is another suggested antilipogenic mechanism of t\textsubscript{10}, c\textsubscript{12} CLA in mammals. Inhibition of transcription [20,21] and activity [22,23] of the stearoyl-CoA desaturase enzyme (SCD1) by t\textsubscript{10}, c\textsubscript{12} CLA have been reported. Loss of SCD1 functionality is believed to result in the lowered fat storage in mammals [24,25]. In yeast the single \(\Delta\alpha\) FA desaturase, Ole1p, is responsible for the monounsaturated FA (MUFA) synthesis [26,27]. The enzyme is strictly controlled by external conditions and nutritional status of the yeast cell to preserve FA homeostasis in membrane lipids [28-30]. The impact of c\textsubscript{9}, t\textsubscript{11} and t\textsubscript{10}, c\textsubscript{12} CLA isomers on transcript abundance of OLE1 was studied by Hokkanen et al. [12] and different effects between the isomers were found: the c\textsubscript{9}, t\textsubscript{11} isomer reduced the transcript abundance of OLE1 whereas the t\textsubscript{10}, c\textsubscript{12} CLA had no effect on its transcription. However, the MUFA content was significantly lower in the t\textsubscript{10}, c\textsubscript{12} CLA treated cell cultures, and the authors suggested that post-transcriptional inhibition of the enzyme occurred in the presence of trans-10 double bond.

The purpose of this research was to find out the conditions which induce TAG degradation by CLA in yeast and whether the enhanced FA oxidation played a role in the lipid reduction. The t\textsubscript{10}, c\textsubscript{12} CLA oxidation has been shown to be degraded efficiently by yeast \(\beta\)-oxidation [31]. In addition we studied, if the t\textsubscript{10}, c\textsubscript{12} CLA isomer could induce TAG degradation in the cells, which had accumulated high TAG content before CLA supplementation. Effect of the t\textsubscript{10}, c\textsubscript{12} isomer on yeast desaturase activity was evaluated by measuring the c\textsubscript{9}, t\textsubscript{11} CLA formation from trans-vaccenic acid. The results showed that the CLA isomers induced lipid degradation after the cells had changed their metabolism from fermentation to respiratory mode. The impact was similar irrespective of the timing of the CLA supplementation. Desaturation of endogenous and exogenously supplemented fatty acids was lowered in the presence of the t\textsubscript{10}, c\textsubscript{12} CLA. Thus, increased lipid degradation and reduced FA desaturation were suggested to be the major lipid reducing effects of the t\textsubscript{10}, c\textsubscript{12} CLA in yeast \textit{S. cerevisiae}.

**Materials and Methods**

**Yeast strain and cultivation conditions**

All FA (t\textsubscript{10}, c\textsubscript{12} CLA; c\textsubscript{9}, t\textsubscript{11} CLA; oleic acid and trans-vaccenic acid, >90% purity) were supplied by Larodan (Sweden). Wild type \textit{S. cerevisiae} strain BY4742 (MAT\textalpha; ura3\Delta0; leu2\Delta0; his3\Delta1; lys2\Delta0; YOR363c::kanMX4) were ordered from Euroscarf. Yeast was precultivated up to OD(600) \textapprox8 in YPD medium (yeast extract 20gL\textsuperscript{-1}, bacterial peptone 20gL\textsuperscript{-1}, glucose 20gL\textsuperscript{-1}) at 30 °C and shaking at 250 rpm and inoculated thereafter to the fresh YPD medium (OD(600) \textapprox0.05). FA were supplemented into the media from the stock solutions (10gL\textsuperscript{-1} in EtOH) to reach a final concentration of 0.3gL\textsuperscript{-1}. If two different FAs were supplemented, the FA concentration also doubled (0.3+0.3gL\textsuperscript{-1}). The control cultivations were supplemented by the same volume of ethanol. Cell samples (5mL) for the lipid analysis were lyophilized and cellular dry weights per volume were determined.

**Extraction of lipids**

Cells from 5mL of culture were resuspended in 0.5mL of dd H\textsubscript{2}O. An equal amount of glass beads (0.5mm diameter) was added and the tubes were vortexed for 45min at room temperature. Lysates were transferred to the glass tubes and the lipids were extracted from the cell debris in 2mL of dichloromethane-methanol (2:1) by shaking vigorously for three hours. Equal volumes (0.5mL) of dichloromethane and 0.1moll\textsuperscript{-1} HCl were added and the tubes were shaken for an additional hour. The samples were centrifuged for 10min at 4500rcf. The lower phase (organic) containing the lipids was collected and dried under a nitrogen stream.

**TAG separation and analysis**

Thin layer chromatography (TLC) was performed to separate lipid classes as described by Jaakola et al. [10]. The lipid sample was dissolved in 200µL of dichloromethane-methanol (100:1) and supplemented with standard-mixture containing...
Desaturation in Yeast

Accelerated TAG degradation was suggested to be the most probable antilipogenic mechanism of t10, c12 CLA in yeast [12]. In this study, we examined further the impact of the CLA isomers on lipid degradation. In particular, we were interested in determining the time point for induction of TAG hydrolysis. Therefore, we grew yeast cells in the presence or absence of CLA and monitored growth, glucose consumption and ethanol consumption, ethanol concentrations reached maximal levels after depletion of glucose, and the column oven (CTO 20A) temperature was 60 °C. Glucose and ethanol were identified and quantified using authentic reagents for external standard calibration using a Class-VP program.

Results and Discussion

Yeast TAG degradation by CLA is induced upon diauxic shift

The highest value, 27mg g⁻¹, of the TAG amount in the control cells increased continuously reaching 24 hours of cultivation and thereafter, the biomass accumulation inhibited growth more than the ethanol levels in all cultures were comparable. The ethanol consumption started after 20 hours of cultivation, ethanol consumption started earlier than that of the control cells. The cultivation. Thus, the metabolic status after the diauxic shift allowed TAG degradation to take place only in the CLA-treated cells. However, neither increased free FA amounts were found nor was the glycerophospholipid content significantly affected by CLA supplementation, indicating utilization of the FA in β-oxidation.

Determination of glucose and ethanol concentrations

Glucose and ethanol were analyzed using a Shimadzu HPLC with the refractive index detector RID-10A. The analytical column used was SP0810 Shodex8.0mm IDx300mm with a Bio-Rad deashing guard column. The eluent was deionized water delivered at a flow rate of 0.7 mL min⁻¹ and the column oven (CTO 20A) temperature was 60 °C. Glucose and ethanol were identified and quantified using authentic reagents for external standard calibration using a Class-VP program.

Figure 1: Glucose and ethanol content of the yeast cultures and TAG FA composition of yeast. The S. cerevisiae cells were grown with CLA isoemers (0.3gL⁻¹) or without FA supplementation (ctrl). Data are averages of two technical replicates of two parallel cultivations. A. Glucose and ethanol content. Filled square, w/o FA supplementation; Filled triangle, cis-9, trans-11 CLA; Filled circle, trans-10, cis-12 CLA, Basic line, glucose content in the medium (gL⁻¹); B. Biomass content. Open square, w/o FA supplementation; Open triangle, cis-9, trans-11 CLA 0.3gL⁻¹; Open circle, trans-10, cis-12 CLA 0.3gL⁻¹. C. Yeast TAG FA content (mg g⁻¹ cdw⁻¹). Black, saturated FA; White, monounsaturated FA; Slashed right, cis-9, trans-11 CLA, Slashed left, trans-10, cis-12 CLA.

TAG synthesis is not inhibited by t10, c12 CLA in yeast

TAG accumulation in t10, c12 CLA fed culture was significantly lower compared to cis 1 CLA treated and control culture as shown in Figure 1C and also as previous studies [10-12]. This...
raised a question, whether the TAG formation was attenuated by the t10, c12 isomer in yeast. Even if the mRNA expression of diacylglycerol acylating enzymes was not inhibited in the t10, c12 CLA-treated culture [12], the inhibition of the enzymatic machinery for TAG formation could not be ruled out in these previous studies. Therefore, we approached the subject by studying, if the t10, c12 CLA could prevent accumulation of other exogenous FA into TAG. This experiment would show whether the TAG biosynthesis is disturbed by the isomer. Furthermore, it was thus far unclear, whether the t10, c12 CLA isomer could induce hydrolysis of TAG if supplemented in the later stages of cultivation. Therefore, we conducted a set of experiments to find whether t10, c12 CLA inhibits lipid accumulation or rather induces degradation of existing lipids.

The tendency of the isomer to inhibit lipid accumulation was studied first. We cultivated yeast with oleic acid alone or in combination with t10, c12 CLA to see, if the incorporation of oleic acid was reduced by the CLA isomer. Oleic acid and t10, c12 CLA were added in equal amounts to the cultivation medium and biomass and TAG accumulation were monitored up to 60 hours. Biomass accumulation of the cultures supplemented with FA exceeded the corresponding biomass accumulation of the control cells (Figure 2A). The highest biomass concentration (8.2 g L-1) was obtained in the culture supplemented with the t10, c12 CLA. Exogenous oleic acid was readily incorporated into TAG which is known to be a way to prevent its lipotoxic effects on membranes [33,34]. In contrast, the t10, c12 CLA accumulated more moderately (Figure 3A), which could indicate, that TAG synthesis was prevented in the presence of the isomer or the uptake of the isomer was slower compared to oleic acid. As shown
in Figure 3A, the t10, c12 CLA lowered the incorporation of oleic acid in TAG fraction. However, the isomer itself incorporated into TAG and total TAG amount was comparable to the levels in the cultivations with oleic acid alone. This proved that TAG accumulation process was not disturbed by the isomer and the enzymes were functioning normally.

The lower accumulation of oleic acid was probably due to the inhibition of yeast endogenous MUFA synthesis in the presence of exogenous unsaturated FA load. Towards the end of the cultivation period, TAG content decreased in the t10, c12 CLA fed cultures indicating the enhanced lipolysis in the presence of CLA after the diauxic shift. TAG degradation offered building blocks for the membranes of newly forming cells and thus the TAG per cell dry weight was drastically lowered. Because of the low incorporation of the CLA isomer in TAG, there were more CLA compared to oleic acid left in the medium for energy source. This was possibly the reason for the faster cell division after diauxic shift in the CLA supplemented cultures. In the oleic acid supplemented culture the new cells synthesized also C16:1. Instead, in the presence of the t10, c12 CLA the MUFA synthesis remained low.

The potential of the isomer to induce TAG degradation in yeast cells, which had been made “obese” by oleic acid feeding during cultivation, was studied next. The control and oleic acid (0.3gL⁻¹) supplemented cultures were cultivated for 24 hours before the supplementation with 0.3gL⁻¹ t10, c12 CLA and we monitored biomass and TAG accumulation. In both cultures, supplementation of t10, c12 CLA induced higher biomass accumulation soon after the supplementation (Figure 2B). Figure 3B shows how the t10, c12 CLA first incorporated in TAG and soon thereafter TAG content started to decrease in the CLA-treated cells. Similarly as in Figure 3A, TAG reduction was apparently due to cell growth which utilized FA liberated from TAG of “old cells” as their membrane components. Partly the increased biomass accumulation could be explained by utilization of the CLA directly from the cultivation medium and partly from the hydrolyzed TAG. Both cultures: cells grown with oleic acid or without FA (control cells), responded similarly to the CLA supplementation. Thus, yeast with normal or high TAG content could be induced to lose lipids by t10, c12 CLA if the isomer was supplemented at later stages of the cultivation.

**TAG reduction by t10, c12 CLA is dependent on yeast FA oxidation capability**

To verify that β-oxidation and TAG reduction by the t10, c12 CLA in yeast were connected, we cultivated the wild type and Δpip2-strains in the presence of the CLA isomers and measured the TAG content of the cells. Figure 4A shows that in the wild type yeast strain TAG amount was lowered due to the t10, c12 CLA treatment after 50 hours of cultivation. Instead, the Δpip2-strain lacking capacity for β-oxidation accumulated TAG comparable to the levels observed in control cells despite the presence of t10, c12 CLA. This indicated that in the presence of t10, c12 CLA TAG reduction was dependent on β-oxidation capacity. Higher biomass accumulation gave support for the enhanced β-oxidation in the t10, c12 CLA fed culture. Interestingly, a higher biomass accumulation was observed also in the deletion strain. However, supplemented CLA served also as a building block for membranes, which saved other carbon resources for growth. Figure 4B shows that t10, c12 CLA was enriched in phospholipids of Δpip2 strain supporting this theory. The c9, t11 isomer inhibited strongly the growth of the Δpip2-strain the lag-phase being over 24 hours (data not shown). Therefore, the lipids of the c9, t11 treated culture couldn’t be measured.

**Δ9 desaturation is attenuated by t10, c12 CLA in yeast**

Previous studies of Hokkanen et al. [12] showed that transcript abundance of OLE1 was not reduced by t10, c12 CLA despite the fact that MUFA amounts were lowered in the t10, c12 CLA-treated cells [10,11] and (Figure 1C, 3 & 4). The desaturase indices: C16:1/C16:0 and C18:1/C18:0, which were calculated for TAG in the experiment, where the cells cultivated with the CLA isomers for 48 hours (Figure 1C) indicated severely inhibited

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MUFA synthesis (Table 1). Trans-vaccenic acid is known to be converted to c9, t11 CLA by yeast Δ9 desaturase Ole1p [35]. Therefore, we compared the formation of c9, t11 CLA from trans-vaccenic acid in the wild type cells and t10, c12 CLA-treated cells to evaluate the effect of the t10, c12 CLA isomer on desaturase activity. As shown in the Figure 5, the cells cultivated with trans-vaccenic acid produced 20mg g⁻¹ c9, t11 CLA isomer during 48 hours. Instead, if the t10, c12 CLA co-supplemented with trans-vaccenic acid, the c9, t11 CLA production was lowered and the cells contained 9mg g⁻¹ c9, t11 CLA. Overall incorporation of trans-vaccenic acid was lower in the t10, c12 CLA-treated cells. This might be due to the reduced capability to convert the potentially detrimental trans-FA to the more favored c9, t1 1 CLA. Because also endogenous MUFA production was significantly lowered, the inhibition of desaturase activity could be concluded to be another mechanism besides induction of lipid degradation by which the t10, c12 CLA reduces lipid content in yeast.

Table 1: Desaturase indexes for C16 and C18 fatty acids in yeast triacylglycerols. The yeast cultures were treated with cis-9, trans-11 or trans-10, cis-12 CLA (0.3 g L⁻¹) or cultivated without fatty acid supplementation (control).

<table>
<thead>
<tr>
<th>Desaturase Index</th>
<th>C16:1:C16:0</th>
<th>C18:1:C18:0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty acid supplemented</td>
<td>w/o (ctrl)</td>
<td>c9, t11 CLA</td>
</tr>
<tr>
<td>13h</td>
<td>2.17±0.00</td>
<td>0.64±0.15</td>
</tr>
<tr>
<td>24h</td>
<td>2.61±0.08</td>
<td>1.34±0.22</td>
</tr>
<tr>
<td>33h</td>
<td>1.81±0.10</td>
<td>1.17±0.09</td>
</tr>
<tr>
<td>50h</td>
<td>3.04±0.32</td>
<td>1.73±0.01</td>
</tr>
</tbody>
</table>

Figure 5: Total FA content of yeast. S. cerevisiae cells were cultivated with trans-vaccenic acid (0.3gL⁻¹), t10, c12 CLA (0.3gL⁻¹) or in combination of both FA (0.3 + 0.3gL⁻¹). Data are averages of two technical replicates of two parallel cultivations. Black, saturated FA; White, monounsaturated FA (C16:1, cis-18:1); Grey, trans-18:1; Slashed right, cis-9, trans-11 CLA, Slashed left, trans-10, cis-12 CLA.

Conclusion

In this study, a connection between metabolic shift and CLA induced lipid degradation was found in yeast S. cerevisiae. Exogenous CLA isomers were incorporated in yeast TAG after supplementation into the medium, but after glucose exhaustion, TAG amount decreased if CLAs were present. The t10, c12 CLA could reduce TAG content independent of the timing of FA supplementation. Biomass accumulation after t10, c12 CLA supplementation indicated that the liberated TAG FA were utilized for energy generation through β-oxidation and as building blocks of newly formed cells resulting in lowered TAG amount per dry cell mass. When t10, c12 CLA was supplemented in the culture after glucose exhaustion, it was apparently utilized directly for energy to produce more biomass. Enhanced β-oxidation by t10, c12 CLA has been proposed to be one of the antilipogenic mechanisms of t10, c12 CLA in mammals. Yeast OaAt1-Pip2 and mammalian PPARα regulate FA oxidation in similar manner and they have been shown to bind to similar ligands [36,37]. The results of this study support the theory of the enhanced β-oxidation by t10, c12 CLA.

In mammals, the PPAR-homologue PPARγ is the transcription factor regulating lipogenesis. The t10, c12 CLA (but not c9, t11 CLA) has been shown to act as an antagonist for PPARγ resulting in lowered lipid accumulation by t10, c12 CLA [4,38]. In yeast the PPAR-type regulation of lipogenesis does not exist and this fact might explain why the TAG accumulation was not inhibited in yeast by CLA. Even though the t10, c12 CLA reduced oleic acid accumulation in TAG, the TAG formation process was not inhibited and lower oleic acid content was more likely resulting from the inhibition of MUFA synthesis by t10, c12 CLA. If t10, c12 CLA was added after the growth phase, the MUFA accumulated comparably to control.

Lower lipid content in the t10, c12 CLA-treated cells was not explained solely by increased lipid degradation. MUFA synthesis
was found to be drastically reduced, if the \( \text{c10}, \text{c12} \) CLA was present. Keeping in mind that desaturase gene transcription was not lowered by the \( \text{c10}, \text{c12} \) CLA [12], the desaturase activity was still shown to be reduced by the isomer evidenced by lowered conversion of trans-vaccenic acid to \( \text{c9}, \text{c11} \) CLA in the \( \text{c10}, \text{c12} \) CLA-treated cells. Desaturase inhibition has been shown previously to have a remarkable role in the fat reducing mechanism of \( \text{c10}, \text{c12} \) CLA also in different mammalian cell types [23,39,40] and was apparently an important antilipogenic mechanism also in yeast.

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