Nutrición Hospitalaria

Original

The combination of resveratrol and CLA does not increase the delipidating effect of each molecule in 3T3-L1 adipocytes

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Abstract

Introduction: Trans-10,cis-12 conjugated linoleic acid (CLA) and resveratrol have been shown to reduce TG content in cultured 3T3-L1 adipocyte acting on different pathways. In recent years, the method of simultaneously targeting several signal transduction pathways with multiple natural products in order to achieve additive or synergistic effects has been tested. However, the combined effect of both molecules on lipid metabolism has not been described before.

Objective: The aim of the present work was to analyze the effect of the combination of trans-10, cis-12 CLA and resveratrol on TG accumulation as well as on FAS, HSL and ATGL expression in 3T3-L1 mature adipocytes, in order to assess a potential interaction between both molecules.

Methods: For this purpose, 3T3-L1 mature adipocytes were treated with the two molecules, both separately and combined, in 10 and 100 μM for 20 hours. TG content and FAS, ATGL and HSL expression were measured by spectrophotometry and Real Time RT-PCR respectively.

Results: Both doses of CLA and 100 M resveratrol decreased TG content in mature adipocytes. The combination of both molecules reduced TG accumulation to the same extent as each one separately. No change in FAS and HSL mRNA levels after CLA and resveratrol treatment was observed. ATGL was not modified by CLA but it was increased by resveratrol and by the combination. This combination did not increase the effect caused by resveratrol on its own.

Conclusion: Lipolysis increase via ATGL is involved in the TG reduction induced by resveratrol and the combination of both molecules. The combination of these two molecules does not increase the efficacy of each molecule separately in mature adipocytes and thus it does not represent an advantage for obesity treatment or prevention.

(*Nutr Hosp.* 2011;26:997-1003)

DOI:10.3305/nh.2011.26.5.5107

Key words: *Resveratrol. CLA. FAS. HSL. ATGL.*

INFLUENCIA DE LOS ÁCIDOS GRASOS OMEGA-3 DE LA LINAZA (LINUM USITATISSIMUM) EN EL DESARROLLO DEL CEREBRO DE RATAS RECIÉN NACIDAS

Resumen

Introducción: Se ha demostrado que el ácido linoleico trans-10, cis-12 conjugado (ALC) y el resveratrol reducen el contenido de TG en el adipocito 3T3-L1 cultivado actuando sobre distintas vías. En los últimos años, se ha probado el método de llegar a diferentes vías de transducción de señal simultáneamente con múltiples productos naturales con el fin de alcanzar efectos aditivos o sinérgicos. Sin embargo, el efecto combinado de ambas moléculas sobre el metabolismo de los lípidos no se ha descrito previamente.

Objetivo: El objetivo de este estudio fue analizar el efecto de la combinación del ALC trans-10, cis-12 y el resveratrol sobre la acumulación de TG así como sobre la expresión de FAS, HSL y ATGL en los adipocitos maduros 3T3-L1 con el propósito de evaluar la interacción potencial entre ambas moléculas.

Métodos: Para este propósito, se trató a adipocitos maduros 3T3-L1 con las dos moléculas, de forma separada y combinada, en 10 y 100 μM durante 20 horas. El contenido de TG y la expresión de FAS, ATGL y HSL se midieron con espectrofotometría y en RT-PCR en tiempo real, respectivamente.

Resultados: ambas dosis de ALC y 100 μM de resveratrol disminuyeron el contenido de TG en los adipocitos maduros. La combinación de ambas moléculas redujo la acumulación de TG en el mismo grado que cada una de ellas por separado. No se observaron cambios en los niveles de ARNm de FAS y HSL tras el tratamiento con ALC y resveratrol. El ATGL no se modificó por el ALC pero se incrementó por el resveratrol y la combinación. Esta combinación no aumentó el afecto causado por el resveratrol solo.

Conclusión: el aumento de la lipólisis vía ATGL está implicado en la reducción de TG inducida por resveratrol y la combinación de ambas moléculas. Esta combinación no aumenta la eficacia de cada una de las moléculas por separado en los adipocitos maduros y, por lo tanto, no representa una ventaja en la prevención ni el tratamiento de la obesidad.

(*Nutr Hosp.* 2011;26:997-1003)

DOI:10.3305/nh.2011.26.5.5107

Palabras clave: *Resveratrol. ALC. FAS. HSL. ATGL.*

Abbreviations

CLA: Conjugated Linoleic Acid.
ATGL: Adipose Triglyceride Lipase.
HSL: Hormone Sensitive Lipase.
FAS: Fatty Acid Syntase.

Introduction

Obesity, a disease characterized by an excessive accumulation of fat in white adipose tissue, is reaching epidemic proportions worldwide and is associated with an increased risk of premature death. The prevalence of obesity in western societies has increased dramatically in recent years. As a result, scientific research is constantly looking for new molecules that could be used as functional ingredients against overweight and obesity. Although numerous molecular targets have been identified, monotherapy has often resulted in a lack of success. Thus, simultaneously targeting several pathways with combinations of natural products to achieve additive or synergistic effects might be an appropriate approach which addresses obesity prevention and/or treatment. The effects of some of these combinations have been reported, namely, ajoene+CLA, resveratrol+genistein, vitamin D+genistein, xanthohumol+guggulsterone, genistein +guggulsterone and vitamin D+guggulsterone.[1-6] Along the same lines, a possible new combination could be CLA and Resveratrol, which have separately demonstrated potential anti-obesity effects.

CLA represents a group of positional and geometric isomers of conjugated dienoic derivatives of linoleic acid. It is present in some dietary sources such as meat and dairy products. It has been shown that CLA reduces TG content in cultured 3T3-L1 adipocyte[7-10] acting on different pathways. A great deal of evidence exists concerning CLA induced lipoprotein lipase activity reduction.[11] By contrast, conflicting results have been reported regarding *de novo* lipogenesis enzyme expression. Although some authors have found a decrease in FAS mRNA levels in 3T3-L1 adipocytes,[12-15] others have observed the opposite effect[16] or even none.[17] With regard to its involvement in lipolysis, CLA treatment has been seen to enhance glycerol release in murine adipocytes.[7,10,18-21] However, further studies are needed to describe its mechanism of action in this pathway.

In recent years a great deal of interest has focussed on resveratrol, a phytoalexin which is well known for its antioxidant effects, but which also has potential effects on lipid metabolism. Resveratrol (3,5,4 -trihydroxystilbene), a naturally occurring dyphenolic compound, is synthesized in a wide variety of plant species in response to stress and fungal infection. It is mostly present in grapes and drinks manufactured from them, such as grape juice or red wine. Resveratrol has been shown to reduce TG content in 3T3-L1 adipocytes[22]

acting on different pathways. It has been described that resveratrol inhibits pre-adipocyte proliferation and maturation[23-25] and that it induces apoptosis and lipolysis in adipocytes.[26,22,27] With regard to *de novo* lipogenesis, it has been described that resveratrol down-regulates the expression of SREBP-1c, FAS and LPL.[26]

Taking all this into account, the aim of the present work was to analyze the effect of the combination of *trans*-10, *cis*-12 CLA and resveratrol on TG accumulation as well as on FAS, HSL and ATGL expression in 3T3-L1 mature adipocytes, in order to assess a potential interaction between both molecules.

Experimental methods

Reagents

Dulbecco's modified Eagle's medium (DMEM) was purchased from GIBCO (BRL Life Technologies, Grand Island, NY). *Trans,10-cis,12* CLA was purchased from Natural Lipids Ltd., (Howdebygda, Norway) and *Trans*-Resveratrol from Cayman Chemicals (Ann Arbor, Michigan, USA). Triglycerides were determined by Infinity Triglycerides reagent (Thermo Electron Corporation, Rockford, IL, USA) and protein concentrations of cell extracts were measured with BCA reagent (Thermo Scientific, Rockford, IL, USA).

Experimental design

The study was carried out in cultured adipocytes. For this purpose a murine cell line of adipocytes, 3T3-L1 adipocytes, was used. Cells were cultured as described below. 3T3-L1 mouse embryo fibroblasts were obtained from American Type Culture Collection (Manassas, VA, USA).

Cell culture

3T3-L1 preadipocytes were cultured in DMEM containing 10% fetal calf serum (FCS). Two days after confluence cells were stimulated to differentiate (day 0) with DMEM containing 10% FCS, 10 µg/mL insulin, 0.5 mM isobutylmethylxanthine (IBMX), and 1 M dexamethasone for 2 days. After day 2, cells were cultured with a differentiation medium containing 10% FCS/DMEM medium and 0.2 µg/mL insulin, which was replaced every two days, for an additional period of 10 days (day 12). At this time > 90% of cells developed mature adipocytes with visible lipid droplets. All media contained 1% Penicillin/Streptomycin (10.000 U/mL), and the medias for differentiation and maturation contained 1% (v/v) of Biotin and Panthothenic Acid. Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

Cell treatment

During the adipogenic phase, on day 12 after differentiation, mature adipocytes grown in 6-well plates were incubated with either 0.1% ethanol (95%) (control group) or with *trans,10-cis,12* CLA and/or resveratrol (10 or 100 M, diluted in 95% ethanol) for 20 hours.

Measurement of triacylglycerol content in adipocytes

After treatment, the medium was removed and cell extracts were used for TG determination. Mature cells were washed extensively with phosphate-buffered saline (PBS) and incubated 3 times with 800 μL of hexane/isopropanol (2:1). The total volume was then evaporated by vacuumed centrifugation and the pellet was resuspended in 200 μL Tritón X-100 in 1% distilled water. Afterwards, TGs were disrupted by sonication and the content was measured by a commercial kit. For protein determinations, cells were lysed in 0.3N NaOH, 0.1% SDS. Protein measurements were performed using the BCA reagent. TG content results were obtained as mmol glycerol/mg protein and have been converted to arbitrary units.

Extraction and analysis of RNA and quantification by Real Time reverse transcription-polymerase chain reaction (Real Time RT-PCR)

RNA samples were extracted using Trizol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The integrity of the RNA extracted from all samples was verified and quantified using a RNA 6000 Nano Assay (Thermo Scientific, Wilmington, DE, USA). RNA samples were then treated with DNase I kit (Applied Biosystems, California, USA) to remove any contamination with genomic DNA.

1.5 μg of total RNA of each sample was reverse-transcribed to first-strand complementary DNA (cDNA) using iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA).

Relative FAS, HSL and ATGL mRNA levels were quantified using Real-Time PCR with an iCycler™- MyiQ™ Real Time PCR Detection System (BioRad, Hercules, CA, USA). β-actin mRNA levels were similarly measured and served as the reference gene. 0.1 L of each cDNA were added to PCR reagent mixture, SYBR Green Master Mix (Applied Biosystems, California, USA), with the upstream and downstream primers (300nM for FAS and 900 nM for HSL and ATGL). Specific primers were synthesized commercially (Tib Molbiol, Berlin, Germany), and the sequences were:

FAS: 5'-AGC CCC TCA AGT GCA CAG TG-3' (forward); 5'-TGC CAA TGT GTT TTC CCT GA-3' (reverse).

HSL: 5'-GGT GAC ACT CGC AGA AGA CAA TA-3' (forward); 5'-GCC GCC GTG CTG TCT CT-3' (reverse).

ATGL: 5'-CAC TTT AGC TCC AAG GAT GA-3' (forward); 5'-TGG TTC AGT AGG CCA TTC CT-3' (reverse).

β-Actin: 5'-ACG AGG CCC AGA GCA AGA G-3' (forward); 5'-GGT GTG GTG CCA GAT CTT CTC-3' (reverse).

The PCR parameters were as follows: initial 2 min at 50°C, denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 30s, annealing at 60°C for 30s and extension at 60°C for 30s. All sample mRNA levels were normalized to the values of -actin and the results expressed as fold changes of threshold cycle (Ct) value relative to controls using the $2^{-\Delta\Delta Ct}$ method[28].

Statistical analysis

Results are presented as mean ± standard error of the mean. Statistical analysis was performed using SPSS 16.0 (SPSS Inc. Chicago, IL, USA). Statistical analysis was determined by ANOVA (Analysis of Variance) followed by Tukey *post hoc* test. Statistical significance was set-up at the $P < 0.05$ level.

Results

Effect of CLA and resveratrol on TG content

TG content was reduced in 3T3-L1 mature adipocytes by CLA at both doses of 10 and 100 M (21.6% and 20.0% respectively). Resveratrol only reduced the TG content in the cells at the higher dose of 100 M (16.6%). When both molecules were combined, 10 M of CLA with 10 M of resveratrol and 100 μM of CLA with 100 M of resveratrol, a decrease of TG content (15.0% and 12.4% respectively) was observed. There was no dose-dependent effect for individual molecules or their combination (fig. 1). Moreover, the combination did not increase the TG content reduction caused by each molecule separately.

Effect of CLA and resveratrol on FAS expression

FAS expression was not modified by CLA or resveratrol at any dose. The combination of both compounds did not result in a significant change of FAS mRNA levels, either at 10 μM or at 100 μM (fig. 2).

Effect of CLA and resveratrol on HSL and ATGL expression

HSL and ATGL expressions were not modified at any CLA dose. Resveratrol treatment did not alter HSL
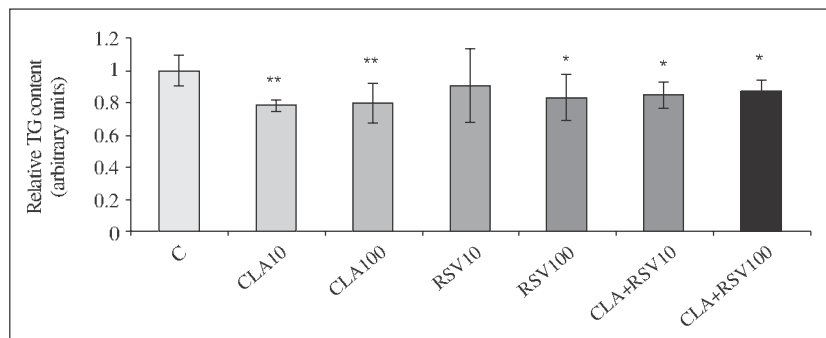
Fig. 1.—TG content in 3T3-L1 cells, after 20 hours of treatment with trans,10-cis,12 CLA and resveratrol, combined or not.
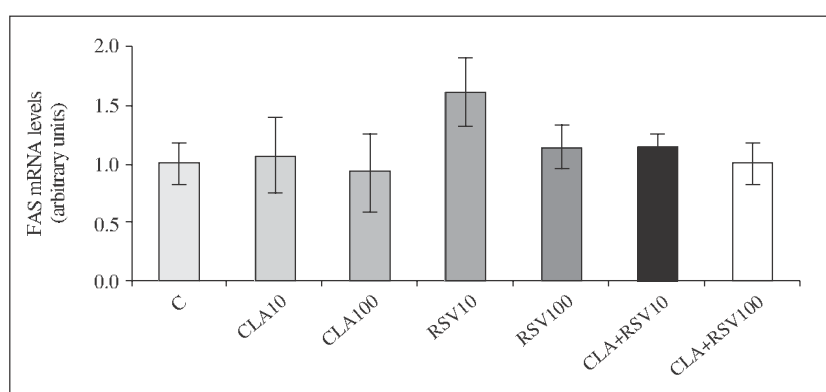

Fig. 2.—FAS mRNA levels in 3T3-L1 cells, after 20 hours of treatment with trans,10-cis,12 CLA and resveratrol, combined or not.

expression but increased ATGL expression at both doses, without a dose-response pattern. As expected, the combination of both molecules at 10 and 100 μM also resulted in no changes in HSL expression. By contrast, this combination increased ATGL expression at both doses without a dose-dependent effect (fig. 3A and 3B). Again, the combination did not increase the effect caused by resveratrol alone.

Discussion

In recent years, targeting several signal transduction pathways simultaneously with multiple natural products, such as ajoene+CLA and resveratrol+genistein, to achieve additive or synergistic effects has been tested.[1,2] To a similar end, we analyzed the effect of the combination of CLA and Resveratrol on TG accumulation because they have separately demonstrated a potential anti-adipogenic effect. There are no data available in the literature concerning the effect of this combination on lipid metabolism. As far as we know, there is only one study of human adipocytes which demonstrates that resveratrol prevents CLA mediated insulin resistance and inflammation.[29]

In the present work, as might be expected, CLA treatment reduced TG content of mature 3T3-L1 adipocytes after 20 hours of treatment. With regard to resveratrol, this polyphenol also reduced TG content at the higher dose. These results are in good accordance with other studies also performed in 3T3-L1 adipocytes under different experimental conditions.[30] However, in the present study, the combination of both compounds did not enhance the effect of each one separately.

The vast majority of other combinations reported in the literature show an additive or synergistic effect. It has been shown with regard to resveratrol that the combination of this molecule and genistein is more potent in exerting antiobesity effects than the individual compounds, by inhibiting adipogenesis, inducing apoptosis and promoting lipolysis in 3T3-L1 adipocytes.[2] Similarly, CLA has been proposed to enhance ajoene-induced apoptosis in mature 3T3-L1 adipocytes by synergistically increasing the expression of several proapoptotic factors.[1] In our study it seems that the magnitude of the reduction reached a plateau with each molecule by itself. Thus, this combination does not favour a greater reduction of lipid content.

In order to gain more insight into the reasons that could explain why this combination did not increase TG content reduction, the expression of a lipogenic enzyme, FAS, and the two main lipases, HSL and ATGL, was measured.
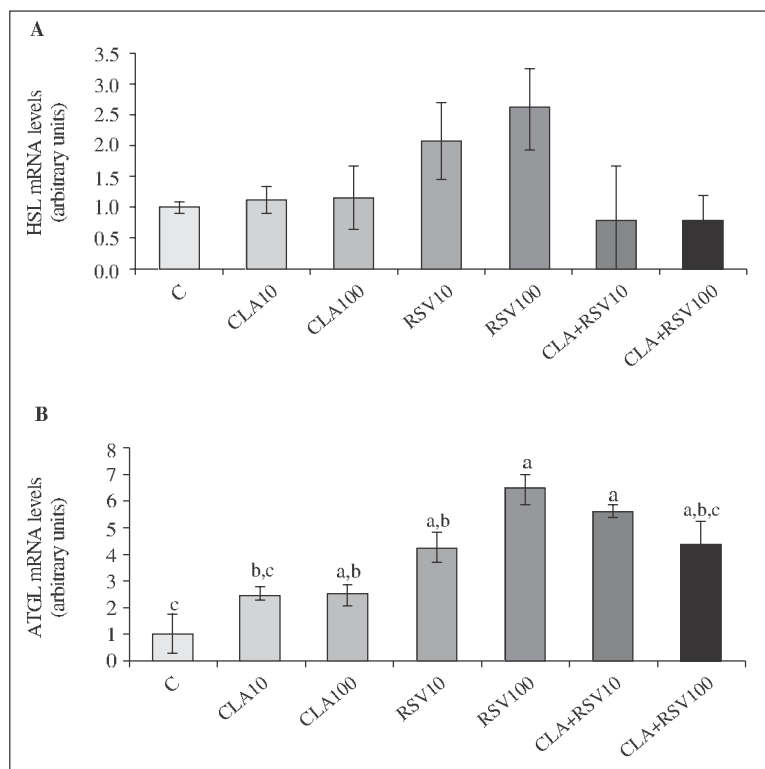
Fig. 3.—HSL (3A) and ATGL (3B) mRNA levels in 3T3-L1 cells, after 20 hours of treatment with trans,10-cis,12 CLA and resveratrol, combined or not.

CLA and resveratrol separately did not affect FAS mRNA expression at any dose after 20 hours of treatment on day 12 of differentiation. Similarly, the combination of both molecules did not alter this parameter. These results do not agree with other published reports. Kang et al.[12] observed a reduction in FAS expression when treating 3T3-L1 maturing cells, from day 0 to 7 of differentiation, with 100 μM trans-10, cis-12 CLA. Rayalam et al.[26] found that, after treating maturing 3T3-L1 preadipocytes with 25 μM of resveratrol, several adipogenic transcription factors and enzymes, such as PPAR and FAS were down-regulated. Similarly, Fischer-Posovszky et al.[31] found a decrease in FAS expression after treating SGBS human adipocytes with 20 to 100 μM of resveratrol during the first four days of differentiation. The difference between these results and data obtained in our study could be related to the day of treatment and the length of the experimental period. While in the present study mature adipocytes were treated on day 12, in the studies mentioned compounds were added during the differentiation of preadipocytes to mature adipocytes. Thus, in those cases the observed effects are a consequence of inhibition of adipocyte maturation, while in our experimental conditions we checked the effect of these compounds directly on mature adipocytes.

Another possible pathway for TG reduction is lipolysis. Thus, the expression of two main lipases, HSL and ATGL, was analyzed. HSL is the most active enzyme against diglycerides (DG), which are hydrolyzed 10 times faster than TG. On the other hand, ATGL selectively performs the first step in TG hydrolysis resulting in the formation of DG and free fatty acids (FFA). Contrarily to HSL, its activity against TG is 10 times more specific than that against DG.

As far as the effects of each molecule on HSL expression were concerned, neither CLA nor resveratrol separately altered HSL mRNA levels. Consequently, a combination of both molecules was without effect. With regard to ATGL expression, while CLA had no effect, resveratrol increased ATGL expression at both doses, and so did the molecule combination.

Most data present in the literature show a stimulation of basal lipolysis by CLA. However little evidence exist concerning its mechanism of action. In an study where lipase expression was analyzed,[32] no significant influence of CLA on HSL and ATGL gene expression was found, but they observed an up-regulation of PKA and perilipin expression, thus, as a whole, lipolysis was stimulated. With regard to resveratrol, our data suggest that resveratrol acts at ATGL level. It must be pointed out that, as far as we are aware, the effect of this polyphenol on HSL gene expression has been analyzed in only one study.[26] They observed that HSL mRNA levels were down-regulated after treating maturing preadipocytes (from day 0 to 6) with 25 μM of resvera-

trol. The difference between this study and ours can be once again explained because of the different day and length of cell treatment.

With regard to the combination of CLA and resveratrol, the only effect observed was an increase in ATGL expression to the same extent as the one caused by resveratrol individually. So, the combination of both molecules did not increase the effect of one of them. Very little information exists in the literature concerning the effect of different molecule combinations on lipolysis. Rayalam et al.[2] observed that neither genistein nor resveratrol separately altered lipolysis, whereas their combination resulted in an enhancement of this pathway. Differences in dose and incubation period between our study and the others might contribute to the varied response of resveratrol and its combinations.

In conclusion, the present work was designed to gain more insight into the effect of *trans*-10, *cis*-12 CLA and resveratrol combination on lipogenesis and lipolysis and its potential contribution to the TG-lowering action in 3T3-L1 cells. The results show that the combination of these two molecules does not increase the efficacy of each molecule separately in mature adipocytes and therefore this does not represent any advantage for obesity treatment or prevention. Lipolysis increase via ATGL is involved in the TG reduction induced by resveratrol and the combination of both molecules. Taking into account that the magnitude of the effects was the same for resveratrol and resveratrol+CLA, it can be stated that the combination of these two molecules does not enhance their individual effect. Thus, it may be suggested that this combination does not represent a useful tool for losing weight.

Acknowledgements

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Effect of resveratrol and trans-10,
cis-12 CLA combination on 3T3-L1
lipid metabolism

Nutr Hosp. 2011;26(5):997-1003

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