

Conjugated linoleic acid-mediated inflammation and insulin resistance in human adipocytes are attenuated by resveratrol

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Abstract Inflammation plays a role in *trans*-10, *cis*-12 (10,12)-conjugated linoleic acid (CLA)-mediated delipidation and insulin resistance in adipocytes. Given the anti-inflammatory role of resveratrol (RSV), we hypothesized that RSV would attenuate inflammation and insulin resistance caused by 10,12 CLA in human adipocytes. RSV blocked 10,12 CLA induction of the inflammatory response by preventing activation of extracellular signal-related kinase and induction of inflammatory gene expression (i.e., IL-6, IL-8, IL-1 β) within 12 h. Similarly, RSV suppressed 10,12 CLA-mediated activation of the inflammatory prostaglandin pathway involving phospholipase A₂, cyclooxygenase-2, and PGF_{2 α} . In addition, RSV attenuated 10,12 CLA increase of intracellular calcium and reactive oxygen species associated with cellular stress, and activation of stress-related proteins (i.e., activating transcription factor 3, JNK) within 12 h. 10,12 CLA-mediated insulin resistance and suppression of fatty acid uptake and triglyceride content were attenuated by RSV. Finally, 10,12 CLA-mediated decrease of peroxisome proliferator-activated receptor γ (PPAR γ) protein levels and activation of a peroxisome proliferator response element (PPRE) reporter were prevented by RSV. RSV increased the basal activity of PPRE, suggesting that RSV increases PPAR γ activity. Collectively, these data demonstrate for the first time that RSV prevents 10,12 CLA-mediated insulin resistance and delipidation in human adipocytes by attenuating inflammation and cellular stress and increasing PPAR γ activity.—Kennedy, A., A. Overman, K. LaPoint, R. Hopkins, T. West, C.-C. Chuang, K. Martinez, D. Bell, and M. McIntosh. **Conjugated linoleic acid-mediated inflammation and insulin resistance in human adipocytes are attenuated by resveratrol.** *J. Lipid Res.* 2009. 50: 225–232.

Supplementary key words stress • anti-inflammatory • delipidation

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Feeding a mixture of conjugated linoleic acid (CLA) isomers [i.e., *trans*-10, *cis*-12 (10,12) CLA and *cis*-9, *trans*-11 (9,11) CLA] reduces adiposity in animals (1) and some humans (2). The triglyceride (TG)-lowering properties of CLA appear to be due exclusively to the 10,12 isomer (3–5), and involve decreased uptake and metabolism of glucose and fatty acids (FA)s (6), and increased lipolysis (7) in adipocytes. These anti-obesity properties of 10,12 CLA are dependent on the activation of mitogen-activated protein kinase/extracellular signal-related kinase (MEK/ERK) (6) and nuclear factor κ B (NF κ B) (8, 9) in adipocytes. These signaling pathways induced by 10,12 CLA are linked to the induction and secretion of cytokines (8, 9), which are known to antagonize peroxisome proliferator-activated receptor γ (PPAR γ) target gene expression and insulin sensitivity (10–15). Consistent with these data, 10,12 CLA supplementation of humans is associated with hyperglycemia, insulin resistance, elevated levels of inflammatory prostaglandins (PGs) and cytokines, and dyslipidemia (16–18).

Recently, supplementation of mice and 3T3-L1 adipocytes with 10,12 CLA has been shown to activate the integrated stress response (ISR) pathway (19), which is linked to inflammation, insulin resistance, and endoplasmic reticulum (ER) stress (20). Cellular stress can be caused by a relatively disproportional influx of macronutrients that adversely affect organelle function, including the mitochondria and ER. This cellular stress increases the release of calcium and reactive oxygen species (ROS), which leads

Abbreviations: AMPK, AMP kinase; ATF3, activating transcription factor 3; [Ca²⁺]_i, intracellular calcium; CLA, conjugated linoleic acid; COX, cyclooxygenase; DCF, dichlorofluorescein; ER, endoplasmic reticulum; ERK, extracellular signal-related kinase; fluo-3 AM, fluo-3 acetoxymethyl ester; ISR, integrated stress response; JNK, c-Jun-NH2-terminal kinase; NF κ B, nuclear factor κ B; PG, prostaglandin; PLA₂, phospholipase A₂; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; ROS, reactive oxygen species; RSV, resveratrol; SIRT1, sirtuin 1; TG, triglyceride; TZD, thiazolidinedione; WAT, white adipose tissue.

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to inflammation and/or insulin resistance (20). These stressors can impair the adipocyte's ability to synthesize and/or store FAs as TG, causing lipids to accumulate in nonadipocytes (e.g., hepatocytes, myotubes) and resulting in disorders like steatosis and insulin resistance, respectively (14). Therefore, it is possible that 10,12 CLA, a *trans*-conjugated FA not normally abundant in the diet, causes cellular stress in adipocytes, initiating a signaling cascade that adversely affects adipocyte function. These issues raise concern about the safe and effective use of supplements containing 10,12 CLA as a dietary strategy for weight loss.

Resveratrol (RSV), a phenolic phytochemical found in grapes, berries, and peanuts, has been shown to inhibit tumor necrosis factor α (TNF α)-induced inflammatory gene expression and secretion in 3T3-L1 adipocytes (21). In mice, supplementing a high-saturated-FA diet with RSV increased lifespan, motor activity, AMP kinase (AMPK), and insulin sensitivity (22) and reduced adipocyte size and metabolic disease (23), compared with a high-saturated-FA diet alone. These and other studies (24) suggest that low micromolar blood levels of RSV protect against inflammatory and stress-related diseases. However, the mechanism(s) through which RSV protects against the development of these diseases are not fully understood, and its impact on CLA-mediated inflammation is unknown.

Therefore, we wanted to determine the extent to which RSV prevented some of the side effects (i.e., inflammation, cellular stress, insulin resistance) associated with CLA supplementation. To begin to answer this question, we examined the isomer-specific influence of CLA in the absence and presence of RSV on 1) the induction or activation of genes, proteins, PGs, ROS, and intracellular calcium levels [Ca^{2+}]_i associated with inflammation and cellular stress, 2) insulin resistance, 3) FA uptake and TG content, and 4) the protein levels and activity of PPAR γ .

MATERIALS AND METHODS

Materials

All cell culture ware was purchased from Fisher Scientific (Norcross, GA). Western Lightning Chemiluminescence Substrate was purchased from Perkin Elmer Life Science (Boston, MA). Immunoblotting buffers and precast gels were purchased from Invitrogen (Carlsbad, CA). DNA-free was purchased from Ambion (Austin, TX). Gene-specific primers were purchased from Applied Biosystems (Foster City, CA). Polyclonal antibodies for anti-GAPDH (sc20357), activating transcription factor 3 (ATF3; sc-188), and β -actin (sc1616) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-P (Ser505) phospholipase A₂ (PLA₂), anti-P (Thr183/185) c-Jun-NH₂-terminal kinase (JNK), and anti-P (Thr-202/204) and total ERK1/2 antibodies were purchased from Cell Signaling Technologies (Beverly, MA). PGF_{2 α} levels were measured in conditioned media using an Enzyme Immunoassay (EIA) kit from Cayman Chemicals (Ann Arbor, MI). FBS was purchased from Hyclone (Logan, UT). The thiazolidinedione (TZD) BRL (rosiglitazone) was a generous gift from Dr. Per Sauerberg, Novo Nordisk, Denmark. Isomers of CLA (+98% pure) were purchased from Matreya (Pleasant Gap, PA) or Natural ASA (Hovdebygd, Norway). The Nucleofector

and Dual Glo luciferase kits were obtained from Amaxa (Cologne, Germany) and Promega (Madison, WI), respectively. Dichlorofluorescein (DCF) and fluo-3 acetoxyethyl ester (fluo-3 AM) were purchased from Molecular Probes (Eugene, OR). All other reagents and chemicals were purchased from Sigma Chemical (St. Louis, MO) unless otherwise stated.

Culturing of human primary adipocytes

Abdominal white adipose tissue (WAT) was obtained from non-diabetic females between the ages of 20 and 50 years with a body mass index ≤ 30 during abdominoplasty. Consent was obtained from the Institutional Review Board at the University of North Carolina at Greensboro. Tissue was digested using collagenase, and stromal vascular cells were isolated as previously described (6). Cultures containing $\sim 50\%$ preadipocytes and $\sim 50\%$ adipocytes were treated between days 6 to 12 of differentiation. Each experiment was repeated at least twice at different times using a mixture of cells from 2–3 subjects unless otherwise indicated.

Preparation of FAs

Both isomers of CLA were complexed to FA-free ($\geq 98\%$) BSA (Sigma #A-7030) at a 4:1 molar ratio using 1 mmol/l BSA stocks. This specific type of BSA has a relatively low inflammatory capacity compared with at least 10 different types of BSA we have tested in our lab.

Immunoblotting

Immunoblotting was conducted as we previously described (6).

Measuring [Ca^{2+}]_i levels

[Ca^{2+}]_i levels were measured using fluo-3 AM. Briefly, cells were preloaded with 5 μM fluo-3 AM and 10% Pluronic F-127, an anionic detergent, at 25°C for 30 min in the dark. Cells were then washed with a buffer consisting of HBSS, CaCl₂, and probenecid, which prevents fluo-3 leakage from cells, and baseline fluorescence was measured using a Synergy Multi-detection Microplate Reader (Bio-Tek, Inc., Winooski, VT). Cells were then treated in the absence or presence of CLA or BSA vehicle in the absence and presence of RSV. Fluorescence was monitored for 10 s intervals for 5 min. Excitation wavelength was 485 nm, and fluorescence was collected at 528 nm. Changes in the ratio of calcium-dependent fluorescence over pre-stimulus background fluorescence (F/F₀) are plotted over time in single representative experiments.

Measuring ROS levels

For the DCF assay, primary human adipocytes were seeded in 96-well plates and differentiated for 6 days. On day 6, media was changed to serum- and phenol red-free media for 24 h. After 24 h, cells were treated with various treatments for 3 h. Cells were then spiked and incubated with 5 μM DCF and kept at 37°C for 1 h. Cells were then washed once with HBSS, and fluorescence was immediately measured in a plate reader with an excitation/emission wavelength of 485/528 nm. DCF values were calculated after normalizing background fluorescence levels of DCF.

Transient transfections of human adipocytes

For measuring PPAR γ activity, primary human adipocytes were transiently transfected with the multimerized peroxisome proliferator response element (PPRE)-responsive luciferase (luc) reporter construct pTK-PPRE3 \times -luc (25) using the Amaxa Nucleofector as previously described (8). On day 6 of differen-

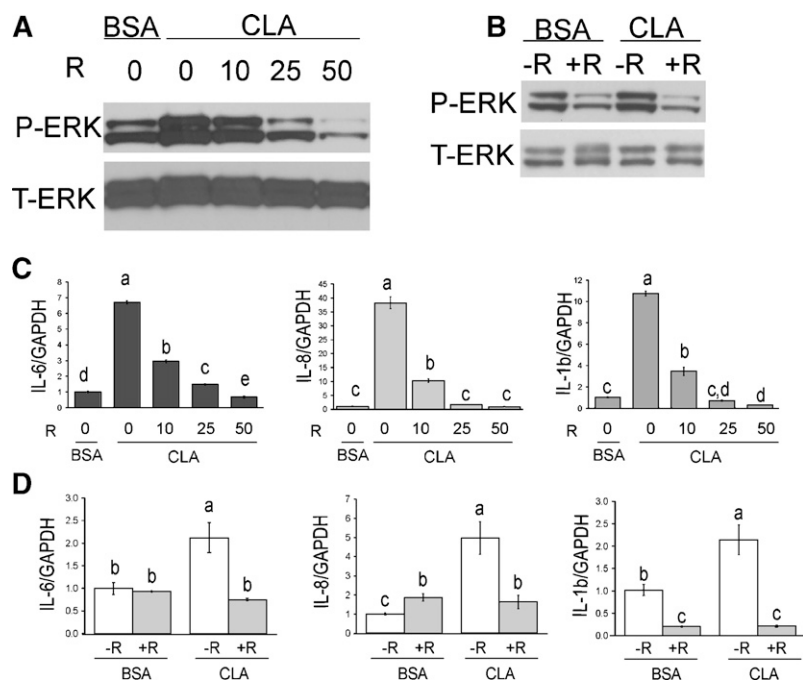


Fig. 1. Resveratrol (RSV) attenuates 10,12-conjugated linoleic acid (CLA) activation of ERK1/2 and induction of cytokines. Cultures of newly differentiated human adipocytes were serum starved for ~ 24 h and then treated for 12 h with BSA vehicle or 50 μM 10,12 CLA in the absence (-) or presence (+) of 0, 10, 25, or 50 μM RSV (R). Subsequently, cultures were harvested for the determination of the protein levels of P-ERK1/2 and total (T)-ERK1/2 by immunoblot (A, B) or mRNA levels for IL-6, IL-8, and IL-1 β by real-time quantitative PCR (qPCR) (C, D). A, B: Data are representative of one (A) or at least three (B) independent experiments. C, D: Data are representative of one (C) or at least three (D) independent experiments. Means (\pm SE; $n = 2$ for C, and $n = 3$ for D) not sharing a lower-case letter differ significantly ($P < 0.05$). The RSV dose in B and D was 50 μM .

tiation, 1×10^6 cells from a 60 mm plate were trypsinized and re-suspended in 100 μl of nucleofector solution (Amaxa) and mixed with 2 μg of pTK-PPRE3 \times -luc and 25 ng pRL-CMV for each sample. Electroporation was performed using the V-33 nucleofector program (Amaxa). Cells were re-plated in 96-well plates after 10 min recovery in calcium-free RPMI media. Firefly luciferase activity was measured using the Dual-Glo luciferase kit and normalized to *Renilla* luciferase activity from the cotransfected control

pRL-CMV vector. All luciferase data are presented as a ratio of firefly luciferase to *Renilla* luciferase activity.

RNA isolation and real-time PCR

Total RNA was isolated from the cultures using Tri Reagent purchased from Molecular Research Center (Cincinnati, OH) according to the manufacturer's protocol. For real-time quantitative

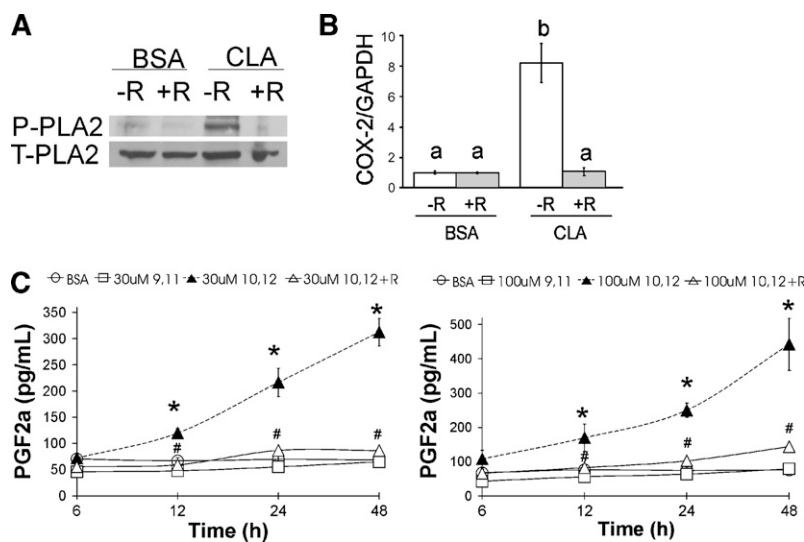


Fig. 2. RSV attenuates 10,12 CLA activation of the inflammatory prostaglandin pathway [A, PLA $_2$; B, cyclooxygenase-2 (COX-2); C, PGF $_{2\alpha}$]. Cultures of newly differentiated human adipocytes were serum starved for ~ 24 h and then treated for either 12 h (A and B) or 6, 12, 24, or 48 h (C) with BSA vehicle or 50 μM 10,12 CLA in the absence (-) or presence (+) of 50 μM RSV (R). Subsequently, cultures were harvested for the determination of the protein levels of P-PLA $_2$ and total (T)-PLA $_2$ by immunoblot (A), mRNA levels for COX-2 by real-time qPCR (B), or the secreted levels of PGF $_{2\alpha}$ in conditioned media by enzyme immunoassay (C). A: Data are representative of at least three independent experiments. B: Means (\pm SE; $n = 3$) not sharing a lower-case letter differ significantly ($P < 0.05$), and are representative of at least three independent experiments. C: Means (\pm SE; $n = 4$) with asterisks (*) differ significantly ($P < 0.05$) from the BSA controls at each time point, and are representative of at least two independent experiments. Means with number sign (#) are significantly lower than cultures treated with 10,12 CLA alone.

PCR (qPCR), 2.0 μg total RNA was converted into first-strand cDNA using Applied Biosystems High-Capacity cDNA Archive Kit. qPCR was performed in an Applied Biosystems 7500 FAST Real Time PCR System using Taqman Gene Expression Assays. To account for possible variation related to cDNA input or the presence of PCR inhibitors, the endogenous reference gene GAPDH was simultaneously quantified for each sample, and data were normalized accordingly.

Lipid staining

Lipid staining was conducted using Oil Red O as previously described (5). The TG levels were measured using a modified, commercially available TG assay as previously described (5). The protein content was determined using the BioRad BCA assay.

2-[^3H]deoxyglucose and [^{14}C]oleic acid uptake

Following the experimental treatments for 48 h, insulin-stimulated uptake of 2-[^3H]deoxyglucose and [^{14}C]oleic acid was measured following a 90 min incubation in the presence of 100 nmol/l human insulin as described previously (6).

Statistical analysis

Statistical analyses were performed for data in Figs. 1D, 2B, 3D, 4A, B, and 5B, C by testing the main effects of CLA (BSA, CLA) and RSV (– or + RSV) and their interaction (CLA \times RSV) using two-way ANOVA (JMP version 6.03; SAS Institute, Cary, NC). For the data shown in Fig. 2C, a two-way ANOVA of the main effects *Treatment* (BSA, 9,11 CLA, 10,12 CLA, 10,12 CLA+RSV) and *Time* (6, 12, 24, 48 h) and their interactions for each dosage was conducted. A one-way ANOVA was conducted for data shown in Figs. 1C, 3B, and 6B, C. Student's *t*-tests were used to compute individual pairwise comparisons of least-square means ($P < 0.05$). Data are expressed as means \pm SE.

RESULTS

RSV blocks 10,12 CLA induction of inflammation and stress-related signaling

A preliminary dose response study showed that 50 μM RSV decreased most effectively the activation of ERK1/2 (Fig. 1A) and JNK (data not shown), and expression of inflammatory genes (Fig. 1C) caused by 10,12 CLA. Thus, we examined the extent to which 50 μM RSV prevented inflammation caused by 50 μM 10,12 CLA in human adipocytes. RSV attenuated 10,12 CLA activation of ERK1/2 (Fig. 1B) and induction of IL-6, IL-8, and IL-1 β gene expression (Fig. 1D) within 12 h. Similarly, RSV blocked 10,12 CLA induction of the inflammatory PG pathway [Fig. 2A, PLA₂; Fig. 2B, cyclooxygenase-2 (COX-2); Fig. 2C, PGF_{2 α}]. Next, we examined the influence of RSV on [Ca^{+2}]_i and ROS levels in CLA-treated cultures. RSV attenuated the rapid, 10,12 CLA increase in [Ca^{+2}]_i and ROS (Fig. 3A, [Ca^{+2}]_i, Fig. 3B, ROS), events directly linked to cellular stress, inflammation, and insulin resistance (20, 26). Similarly, RSV attenuated 10,12 CLA activation of the stress-related proteins ATF3 and JNK (Fig. 3C), and the mRNA levels of ATF3 (Fig. 3D). RSV alone increased the levels of ATF3 mRNA and protein. Taken together, these data demonstrate that RSV attenuates 10,12 CLA-mediated inflammation and cellular stress in cultures of human adipocytes.

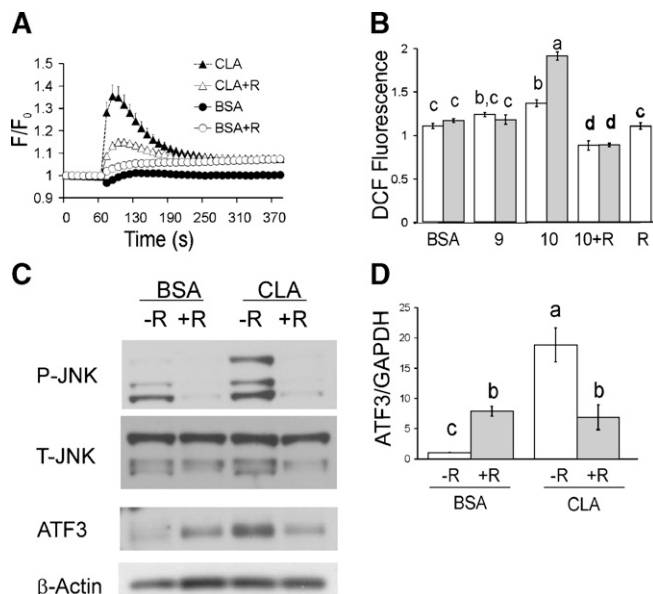


Fig. 3. RSV attenuates 10,12 CLA increase in intracellular calcium and indicators of cellular stress. **A:** Cultures of newly differentiated human adipocytes were serum starved for ~ 24 h and then preloaded with 5 μM fluo-3 acetoxymethyl ester. Subsequently, cultures were injected with 30 μM 10,12 CLA (closed triangle), 30 μM 10,12 CLA + 50 μM RSV (CLA+R, open triangle), BSA vehicle (BSA, closed circle), or BSA+RSV (BSA+R, open circle). Emitted fluorescence intensities were collected over time using a multi-detection microplate reader. Excitation wavelength was 485 nm and fluorescence was collected at 528 nm. Means (\pm SE; $n = 12$) are representative of three independent experiments. **B:** Cultures of newly differentiated human adipocytes were serum starved for ~ 24 h and then treated with 50 μM (open bar) or 150 μM (closed bar) BSA, 9,11 CLA(9), 10,12 CLA(10), 10,12 CLA + 50 μM RSV (10+R), or 50 μM RSV alone(R) for 3 h. Cultures were then loaded with dichlorofluorescein (DCF) for 1 h, and emitted fluorescence intensities were measured using a multi-detection microplate reader. Excitation wavelength was 485 nm and fluorescence was collected at 528 nm. Means (\pm SE; $n = 3$ –12) are representative of three independent experiments. **C, D:** Cultures of newly differentiated human adipocytes were serum starved for ~ 24 h and then treated for 12 h with BSA vehicle or 50 μM 10,12 CLA in the absence (–) or presence (+) of 50 μM RSV (R). Subsequently, cultures were harvested for the determination of the protein levels of P-JNK, total (T)-JNK, activating transcription factor 3 (ATF3), and β -actin by immunoblot (C) or mRNA levels for ATF3 by real-time qPCR (D). **C:** Data are representative of at least three independent experiments. **D:** Means (\pm SE; $n = 3$) not sharing a lower-case letter differ significantly ($P < 0.05$), and are representative of at least two independent experiments.

10,12 CLA suppression of insulin sensitivity, FA uptake, and TG content are prevented by RSV

We previously demonstrated that 10,12 CLA causes insulin resistance, dependent on the activation of ERK1/2 (6) and NF κ B (8). Given the insulin-sensitizing effects reported for RSV in rodents (22, 23, 27), we speculated that RSV would improve insulin sensitivity in cultures of human adipocytes treated with 10,12 CLA. Indeed, 10,12 CLA-treated cultures co-supplemented with 50 μM RSV had higher levels of insulin-stimulated glucose uptake, compared with cultures treated with 10,12 CLA alone (Fig. 4A). Consistent with these data, RSV blocked 10,12 CLA induction of SOCS-3, a protein that causes insulin resistance through

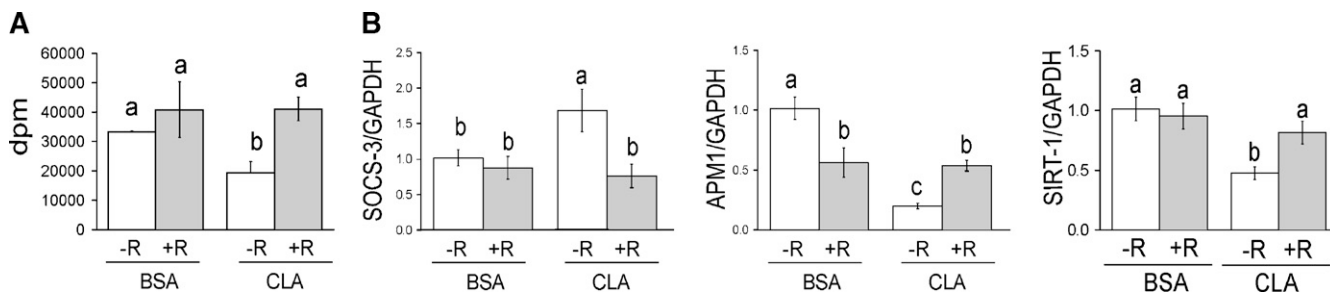


Fig. 4. RSV blocks 10,12 CLA-mediated insulin resistance. Cultures of newly differentiated human adipocytes were serum starved for ~ 24 h and then treated for either 48 h (A) or 24 h (B) with BSA vehicle or 50 μM 10,12 CLA in the absence (–) or presence (+) of 50 μM RSV (R). A: Insulin-stimulated glucose uptake using 2-[^3H]deoxyglucose was measured after a 90 min incubation in the presence of insulin. Means ($\pm\text{SE}$; $n = 6$) not sharing a lower-case letter differ significantly ($P < 0.05$). B: The mRNA levels of suppressor of SOCS-3, adiponectin (APM-1), and sirtuin 1 (SIRT-1) were measured by real-time qPCR. Means ($\pm\text{SE}$; $n = 3$) not sharing a lower-case letter differ significantly ($P < 0.05$) and are representative of at least two independent experiments.

serine phosphorylation of IRS-1. RSV also attenuated 10,12 CLA suppression of adiponectin and sirtuin 1 (SIRT1) mRNA levels, compared with cultures treated with 10,12 CLA alone, suggesting that RSV enhances glucose and/or FA metabolism in these cultures (Fig. 4B). Similarly, RSV co-supplementation attenuated delipidation and suppression of FA uptake by 10,12 CLA (Fig. 5A–C). Thus, RSV may enhance insulin-stimulated glucose and FA uptake in CLA-treated cultures by upregulating genes that stimulate metabolism.

10,12 CLA suppression of PPAR γ inhibited by RSV

To determine the mechanism by which RSV improves insulin sensitivity and reduces delipidation, we examined the extent to which RSV enhanced the protein levels and activity of PPAR γ , a transcription factor that enhances glucose and FA uptake and utilization. RSV prevented CLA-mediated decrease of PPAR γ protein levels (Fig. 6A). To determine whether RSV blocks CLA suppression of PPAR γ activity, the ligand-induced activation of a PPRE-luciferase reporter construct was measured. 10,12 CLA-mediated suppression of the BRL-activated PPRE reporter was prevented by co-supplementation with RSV (Fig. 6B), suggesting that RSV prevents CLA suppression of ligand-

stimulated PPAR γ activity. Concordantly, RSV increased the activation of the PPRE reporter in a dose-dependent manner in the absence of BRL (Fig. 6C). Collectively, these data support our hypothesis that RSV enhances insulin sensitivity and TG content of 10,12 CLA-treated human adipocytes by increasing the activity and/or protein expression of PPAR γ .

DISCUSSION

Feeding mixed isomers of CLA, or 10,12 CLA alone, has been shown to reduce body fat and the TG content of adipocytes, especially in murine models (1). However, adverse metabolic complications (i.e., inflammation, ISR, insulin resistance) have been reported with CLA supplementation of humans, particularly for the 10,12 isomer (16–19). In this article, we demonstrate for the first time that RSV, a phytoalexin with antioxidant properties, attenuates markers of inflammation, cellular stress, ROS production, insulin resistance, and delipidation in cultures of newly differentiated human adipocytes treated with 10,12 CLA. Central to this mechanism is our discovery that RSV blocks 10,12 CLA-mediated increase in [Ca^{+2}], which is essential

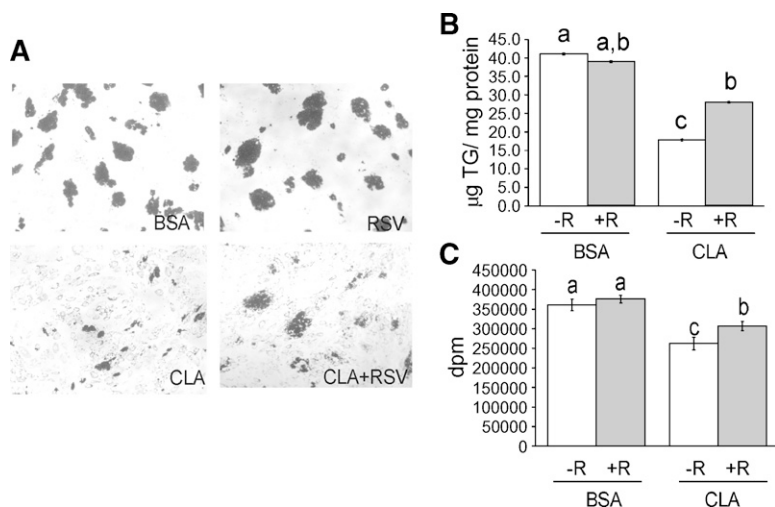


Fig. 5. RSV attenuates delipidation by 10,12 CLA. Cultures of newly differentiated human adipocytes were treated in adipocyte media for 7 days with BSA vehicle or 50 μM 10,12 CLA in the absence (–) or presence (+) of 50 μM RSV (R). Fresh media containing treatments were changed every 2 days. Cultures were then either stained with Oil Red O and phase-contrast photomicrographs were taken using an Olympus inverted microscope with a 10 \times objective (A) or their triglyceride (TG) content was measured using a commercially available TG assay kit (B). A: Data are representative of two independent experiments. B: Means ($\pm\text{SE}$; $n = 6$) not sharing a lower-case letter differ significantly ($P < 0.05$). C: Cultures of newly differentiated human adipocytes were serum starved for ~ 24 h and then treated for 48 h with BSA vehicle or 50 μM 10,12 CLA in the absence (–) or presence (+) of 50 μM RSV (R). [^{14}C]oleic acid uptake was measured after 90 min incubation in the presence of insulin. Means ($\pm\text{SE}$; $n = 3$) not sharing a lower-case letter differ significantly ($P < 0.05$).

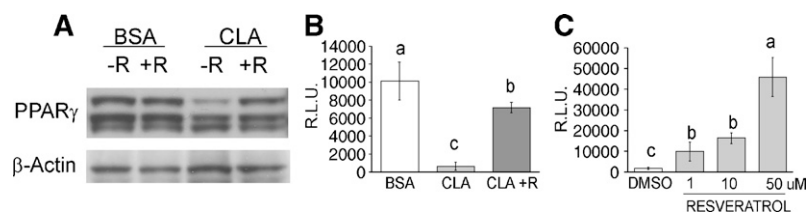


Fig. 6. RSV inhibits 10,12 CLA suppression of peroxisome proliferator-activated receptor γ (PPAR γ) activity. Cultures of newly differentiated human adipocytes were serum starved for ~ 24 h and then treated for 24 h with BSA vehicle or 50 μ M 10,12 CLA in the absence (–) or presence (+) of 50 μ M RSV (R). Subsequently, cultures were harvested for the determination of PPAR γ and β -actin protein levels by immunoblot (A). B: Cultures of newly differentiated human adipocytes were transfected on day 6 with pTK-PPRE3 \times -Luc and pRL-CMV. Transfected cells were treated with BSA vehicle, 50 μ M 10,12 CLA, or 50 μ M 10,12 CLA + 50 μ M RSV (R) for 24 h. Subsequently, cultures were treated with 0.1 μ M BRL for 28 h, and then the luciferase activation of the reporter was measured using a luminometer (\pm SE; $n = 3$). C: Transfected cells were treated with 1, 10, or 50 μ M RSV for 24 h, and then the luciferase was measured as in B (\pm SE; $n = 3$). Means (\pm SE; $n = 3$) not sharing a lower-case letter differ significantly ($P < 0.05$). Data in A–C are representative of at least two independent experiments.

for CLA-mediated inflammation and cellular stress (28), and 2) suppression of PPAR γ activity in primary cultures of human adipocytes treated with 10,12 CLA.

On the basis of these and our previously (un)published data, we propose the following working model (Fig. 7) by which RSV prevents 10,12 CLA-mediated inflammation (Figs. 1, 2), cellular stress (Fig. 3), insulin resistance (Fig. 4), and, ultimately, delipidation (Fig. 5). We speculate that RSV initially blocks CLA-mediated ROS production or accumulation, and release of calcium from the ER. This prevents cellular stress initiated by ROS and calcium signaling. Without these signals to activate MAPKs (e.g., ERK, JNK) and other inflammatory proteins (e.g., NF κ B, COX-2, PLA $_2$) or PGs (e.g., PGF $_{2\alpha}$), 1) inflammatory cytokines (e.g., IL-6, IL-1 β) and chemokines (IL-8) are not induced, 2) insulin signaling is not disrupted, and 3) the protein levels and activity of PPAR γ and the TG levels are preserved (Figs. 5, 6). This allows for normal insulin signaling, glucose and FA uptake and metabolism, and TG accumulation in adipocytes.

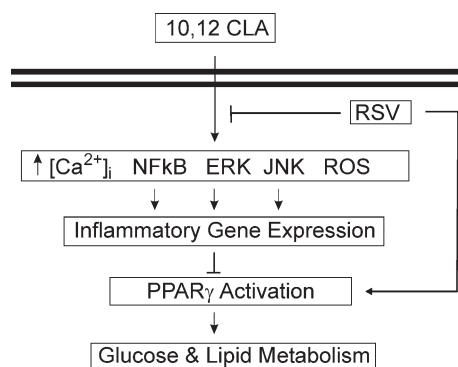


Fig. 7. Working model. RSV initially blocks 10,12 CLA-mediated increase in the levels of reactive oxygen species (ROS) and intracellular calcium ($[Ca^{2+}]_i$), thereby preventing ROS and calcium signaling. Without these signals to activate ERK, JNK, nuclear factor κ B (NF κ B), inflammatory cytokines, and prostaglandins, PPAR γ activity is not suppressed. This allows for normal insulin signaling, glucose and FA uptake and metabolism, and TG accumulation in adipocytes. RSV may also directly activate PPAR γ .

Consistent with our data in human adipocytes, 50–150 μ M RSV has been shown in vitro to reduce inflammation in murine 3T3-L1 adipocytes (21), to reduce oxidative stress in human lung epithelial cells (29, 30), to reduce ER stress in mouse macrophages (31), to decrease TNF α -mediated NF κ B activation in hepatocytes (32) and coronary arterial endothelial cells (33), and to enhance glucose transport in muscle (34). Thus, RSV reduces inflammation and enhances glucose and FA utilization in vivo and in vitro, although the mechanism is unknown.

Further support for our working model comes from studies showing that RSV decreases $[Ca^{2+}]_i$ levels following stimulation with various inflammatory agents or disease states. For example, elevated levels of $[Ca^{2+}]_i$ induced by severe acute pancreatitis were attenuated by RSV (35). Similarly, RSV reduced $[Ca^{2+}]_i$ levels in stress-induced oxygen-glucose deprivation/reperfusion in primary neurons of neonatal rats (36). Finally, *trans*-RSV prevented platelet aggregation by inhibiting elevated $[Ca^{2+}]_i$ levels (37). Thus, RSV suppression of the levels of ROS and $[Ca^{2+}]_i$ is an important mechanism through which RSV prevents these deleterious side effects of 10,12 CLA.

Alternatively, RSV may suppress inflammation by activating PPAR γ . Support for this hypothesis comes from studies showing that RSV activates PPAR γ in CaCo2 cells (38) and macrophages (39). Additionally, our data show that RSV robustly induces PPAR γ activity and prevents CLA-mediated suppression of TZD-induced PPAR γ activation (Fig. 6). Further support comes from studies showing that compounds that enhance PPAR γ activity and insulin sensitivity antagonize NF κ B-mediated signaling, and vice versa (10, 11, 40, 41). For example, TZDs, which are high-affinity PPAR γ ligands, suppress inflammation (40, 41). In contrast, PPAR γ depletion via RNA interference enhances the inflammatory responses of TNF α (42). Consistent with these data, rosiglitazone prevents CLA-mediated insulin resistance and hepatic steatosis in rats (14, 15) and delipidation of human adipocytes (13).


One proposed mechanism for the anti-inflammatory actions of PPAR γ ligands is via (trans)repression of inflammatory gene transcription (43–46). Activation of PPAR γ

can repress the transcriptional activation of inflammatory genes by 1) direct interaction with the mediator of transcription (i.e., NFκB, JNK), 2) inhibition of its co-activator recruitment, or 3) inhibition of its co-repressor clearance. For an example of transrepression, ligand-activated PPARγ becomes SUMOylated, and then binds to co-repressor complexes at inflammatory gene promoters, thereby inhibiting dismissal of nuclear receptor corepressor/histone deacetylase NCoR/HDAC, which blocks inflammatory gene transcription (43, 44). Notably, this pathway does not interfere with transactivation of PPARγ-responsive genes (43, 44). Studies are under way to determine whether this is the mechanism through which RSV prevents inflammation in CLA-treated adipocytes.

RSV has also been shown to enhance insulin sensitivity and glucose uptake in C2C12 myotubes by enhancing AMPK activity (40). AMPK is activated by adiponectin, and by SIRT1 and PGC1α (23, 27). These events are directly linked to mitochondrial biogenesis and oxidative metabolism, which are positively regulated by PPARα. Consistent with these data, RSV prevented or attenuated insulin resistance and the suppression of SIRT1 and adiponectin mRNA levels in CLA-treated adipocytes (Fig. 4). This effect of RSV may be responsible for increasing glucose and FA uptake for oxidative metabolism, which we have previously shown is suppressed by 10,12 CLA (6). However, we do not know whether this was due to increased SIRT1 or PGC-1α activation by RSV.

SIRT1 activation appears to be an important means through which RSV enhances glucose and FA utilization, at least in muscle. SIRT1 increases the activation of PGC-1α and FOXO1-C, thereby increasing mitochondrial biogenesis and oxidative metabolism (47). Furthermore, several studies have demonstrated that feeding 20–400 mg/kg body weight/day of RSV prevents insulin resistance and adiposity in mice fed a high-fat diet (22, 23). These studies suggest that RSV shifts excess calories away from storage in WAT and toward oxidation in muscle and brown adipose tissue, in part by activating SIRT1. In contrast to our hypothesis that RSV activates PPARγ, SIRT1 overexpression in murine adipocytes decreased PPARγ activity, and treatment of murine adipocytes with 50–100 μM RSV enhanced FA mobilization and release. However, PPARγ activities in WAT of mice or in cultures of adipocytes treated with RSV were not investigated (48). Thus, SIRT1 activation by RSV is clearly linked to enhanced glucose and FA metabolism in muscle, and involves AMPK activation. However, the effects of RSV on SIRT1 activation and adipocyte metabolism are still unclear, and differences in SIRT1 regulation by RSV between mouse and human adipocytes are unknown.

Taken together, these data demonstrate that RSV prevents 10,12 CLA-mediated inflammation, insulin resistance, and delipidation of human adipocytes. Potential anti-inflammatory mechanisms for RSV include preventing CLA-mediated ROS accumulation and release of calcium from the ER, which are associated with cellular stress and inflammation, and antagonism of PPARγ activity. Alternatively, RSV may be directly activating PPARγ, which

has the potential to (trans)repress inflammatory gene transcription. Studies are under way to examine these proposed mechanisms. 

REFERENCES

- House, R., J. Cassady, E. Eisen, M. McIntosh, and J. Odle. 2005. Conjugated linoleic acid evokes delipidation through the regulation of genes controlling lipid metabolism in adipose tissue and liver. *Obes. Rev.* **6**: 247–258.
- Whigman, L., A. Watras, and D. Schoeller. 2007. Efficacy of conjugated linoleic acid for reducing fat mass: a meta-analysis in humans. *Am. J. Clin. Nutr.* **85**: 1203–1211.
- Park, Y., J. Storkson, K. Albright, W. Liu, and M. Pariza. 1999. Evidence that trans-10, cis-12 isomer of conjugated linoleic acid induces body composition changes in mice. *Lipids.* **34**: 235–241.
- Brown, J. M., Y. D. Halverson, R. Lea-Currie, C. Geigerman, and M. McIntosh. 2001. Trans-10, cis-12, but not cis-9, trans-11, conjugated linoleic acid attenuates lipogenesis in primary cultures of stromal vascular cells from human adipose tissue. *J. Nutr.* **131**: 2316–2321.
- Brown, M., M. Sandberg-Boysen, S. Skov, R. Morrison, J. Storkson, R. Lea-Currie, M. Pariza, S. Mandrup, and M. McIntosh. 2003. Isomer-specific regulation of metabolism and PPARγ by conjugated linoleic acid (CLA) in human preadipocytes. *J. Lipid Res.* **44**: 1287–1300.
- Brown, J. M., M. Sandberg-Boysen, S. Chung, O. Fabiyi, R. Morrison, S. Mandrup, and M. McIntosh. 2004. Conjugated linoleic acid (CLA) induces human adipocyte delipidation: autocrine/paracrine regulation of MEK/ERK signaling by adipocytokines. *J. Biol. Chem.* **279**: 26735–26747.
- Chung, S., J. M. Brown, and M. McIntosh. 2005. Trans-10, cis-12 CLA increases adipocyte lipolysis and alters lipid droplet-associated proteins: role of mTOR and ERK signaling. *J. Lipid Res.* **46**: 885–895.
- Chung, S., J. M. Brown, J. N. Provo, R. Hopkins, and M. McIntosh. 2005. Conjugated linoleic acid promotes human adipocyte insulin resistance through NFκB-dependent cytokine production. *J. Biol. Chem.* **280**: 38445–38456.
- Poirier, H., J. Shapiro, R. Kim, and M. Lazar. 2006. Nutritional supplementation with trans-10, cis-12 conjugated linoleic acid induces inflammation of white adipose tissue. *Diabetes.* **55**: 1634–1641.
- Suzawa, M., I. Takada, J. Yanagisawa, F. Ohtake, S. Ogawa, T. Yamauchi, T. Kadowaki, Y. Takeuchi, H. Shibuya, Y. Gotoh, et al. 2003. Cytokines suppress adipogenesis and PPAR-γ function through the TAK1/TAB1/NIK cascade. *Nat. Cell Biol.* **5**: 224–230.
- Adams, M., M. Reginato, D. Shao, M. Lazar, and V. Chatterjee. 1997. Transcriptional activation by peroxisome proliferator activated receptor gamma is inhibited by phosphorylation at a consensus mitogen-activated protein kinase site. *J. Biol. Chem.* **272**: 5128–5132.
- Chung, S., K. LaPoint, A. Kennedy, K. Martinez, M. Boysen-Sandberg, and M. McIntosh. 2006. Preadipocytes mediate lipopolysaccharide-induced inflammation and insulin resistance in primary cultures of newly differentiated human adipocytes. *Endocrinology.* **147**: 5340–5351.
- Kennedy, A., S. Chung, K. LaPoint, O. Fabiyi, and M. McIntosh. 2008. Trans-10, cis-12 conjugated linoleic acid antagonizes ligand-dependent PPARγ activity in primary cultures of human adipocytes. *J. Nutr.* **138**: 455–461.
- Lui, L-F., A. Purushotham, A. Wendel, and M. Belury. 2007. Combined effects of rosiglitazone and conjugated linoleic acid on adiposity, insulin sensitivity, and hepatic steatosis in high fat-fed mice. *Am. J. Physiol.* **292**: G1671–G1682.
- Purushotham, A., A. Wendel, L-F. Lui, and M. Belury. 2007. Maintenance of adiponectin attenuates insulin resistance induced by dietary conjugated linoleic acid. *J. Lipid Res.* **48**: 444–452.
- Riserus, U., S. Basu, S. Jovinge, G. Fredrickson, J. Arnlov, and B. Vessby. 2002. Supplementation with conjugated linoleic acid causes isomer-dependent oxidative stress and elevated C-reactive protein. *Circulation.* **106**: 1925–1929.
- Riserus, U., P. Arner, K. Brismar, and B. Vessby. 2002. Treatment with dietary trans-10 cis-12 conjugated linoleic acid causes isomer-specific insulin resistance in obese men with the metabolic syndrome. *Diabetes Care.* **25**: 1516–1521.
- Tholstrup, T., M. Raff, E. Straarup, P. Lund, J. Bruun, and S. Basur. 2007. Opposing effects of trans-10, cis-12 conjugated linoleic acid

- and cis-9, trans-11 conjugated linoleic acid on risk markers for coronary heart disease. *FASEB J.* **21**: 701A, A728.
19. LaRosa, P., J. Riethoven, H. Chen, Y. Xia, Y. Xhou, M. Chen, J. Miner, and M. Fromm. 2007. Trans-10, cis-12 conjugated linoleic acid activates the integrated stress response pathway in adipocytes. *Physiol. Genomics.* **31**: 544–553.
 20. Gregor, M., and G. Hotamisligil. 2007. Adipocyte stress: the endoplasmic reticulum and metabolic disease. *J. Lipid Res.* **48**: 1905–1914.
 21. Ahn, J., H. Lee, S. Kim, and T. Ha. 2007. Resveratrol inhibits TNF α -induced changes in adipokines in 3T3-L1 adipocytes. *Biochem. Biophys. Res. Commun.* **364**: 972–977.
 22. Baur, J., K. Pearson, N. Price, H. Jamieson, C. Lerin, A. Kalra, V. V. Prabh, J. S. Allard, G. Lopez-Lluch, K. Lewis, et al. 2006. Resveratrol improves health and survival of mice on a high-calorie diet. *Nature.* **444**: 337–342.
 23. Lagouge, M., C. Argmann, Z. Gerhart-Hines, H. Meziane, C. Lerin, F. Dausin, N. Messadeq, J. Milne, P. Lambert, P. Elliott, et al. 2006. Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1 α . *Cell.* **127**: 1109–1122.
 24. Saiko, P., A. Szakmary, W. Jaeger, and T. Szekeres. 2008. Resveratrol and its analogs: defense against cancer, coronary disease and neurodegenerative maladies or just a fad? *Mutat. Res.* **658**: 68–94.
 25. Kliewer, S., K. Umesono, D. Noonan, R. Heyman, and R. Evans. 1992. Convergence of 9-cis retinoic acid and peroxisome proliferators signaling pathways through heterodimer formation of their receptors. *Nature.* **358**: 771–774.
 26. Houstis, N., E. Rosen, and E. Lander. 2006. Reactive oxygen species have a causal role in multiple forms of insulin resistance. *Nature.* **440**: 944–948.
 27. Sun, C., F. Zhang, X. Ge, T. Yan, X. Chen, X. Shi, and Q. Zhai. 2007. SIRT1 improves insulin sensitivity under insulin resistance conditions by repressing PTP1B. *Cell Metab.* **6**: 307–319.
 28. Kennedy, A., S. Chung, R. Hopkins, K. LaPoint, and M. McIntosh. 2007. Inflammation and delipidation induced by trans-10, cis-12 conjugated linoleic acid (CLA) are linked to intracellular calcium accumulation in primary cultures of human adipocytes (Abstract). *FASEB J.* **21**: 703.
 29. Kode, A., S. Rajendrasozhan, S. Caito, S. Yang, L. Megson, and I. Rahman. 2008. Resveratrol induces glutathione synthesis by activation of Nrf2 and protects against cigarette smoke-mediated oxidative stress in human lung epithelial cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **294**: L478–L488.
 30. Robb, E., M. Page, B. Wiens, and J. Stuart. 2008. Molecular mechanisms of oxidative stress induced by resveratrol: specific and progressive induction of MnSOD. *Biochem. Biophys. Res. Commun.* **376**: 406–412.
 31. Tabata, Y., K. Takano, T. Ito, M. Iinuma, T. Yoshimoto, H. Miura, Y. Kitao, S. Ogawa, and O. Hori. 2007. Vaticanol B, a resveratrol tetramer, regulates endoplasmic stress and inflammation. *Am. J. Physiol. Cell Physiol.* **293**: C411–C418.
 32. Yu, H., C. Pan, S. Zhao, Z. Wang, H. Zhang, and W. Wu. 2008. Resveratrol inhibits tumor necrosis factor- α -mediated matrix metalloproteinase-9 expression and invasion of human hepatocellular carcinoma cells. *Biomed. Pharmacol.* **62**: 366–372.
 33. Csiszar, A., K. Smith, N. Labinsky, Z. Orosz, A. Rivera, and Z. Ungvari. 2006. Resveratrol attenuates TNF- α induced activation of coronary arterial endothelial cells: role of NF- κ B inhibition. *Am. J. Physiol.* **291**: H1694–H1699.
 34. Park, C., M. Kim, H. Lee, B. Min, H. Bae, W. Choe, S. Kim, and J. Ha. 2007. Resveratrol stimulates glucose transport in C2C12 myotubes by activating AMP-activated protein kinase. *Exp. Mol. Med.* **39**: 222–229.
 35. Wang, L., Q. Ma, X. Chen, H. Sha, and Z. Ma. 2008. Effects of resveratrol on calcium regulation in rats with severe acute pancreatitis. *Eur. J. Pharmacol.* **580**: 271–276.
 36. Gong, Q. H., Q. Wang, J. S. Shi, X. N. Huang, Q. Liu, and H. Ma. 2007. Inhibition of caspases and intracellular free Ca²⁺ concentrations are involved in resveratrol protection against apoptosis in rat primary neuron cultures. *Acta Pharmacol. Sin.* **28**: 1724–1730.
 37. Dobrydneva, Y., R. Williams, and P. Blackmore. 1999. trans-Resveratrol inhibits calcium influx in thrombin-stimulated human platelets. *Br. J. Pharmacol.* **128**: 149–157.
 38. Ulrich, S., S. Loitsch, O. Rau, A. Von Knethen, B. Brune, M. Zsilavec, and J. Stein. 2006. Peroxisome proliferators-activated receptor gamma as a molecular target of resveratrol-induced modulation of polamine metabolism. *Cancer Res.* **66**: 7348–7354.
 39. Ge, H., J. F. Zhang, B. S. Guo, Q. He, B. Y. Wang, B. He, and C. Q. Wang. 2006. Resveratrol inhibits macrophage expression of EMMPRIN by activating PPAR γ . *Vascul. Pharmacol.* **46**: 114–121.
 40. Ruan, H., H. Pownall, and H. Lodish. 2003. Troglitazone antagonizes tumor necrosis factor- α induced reprogramming of adipocyte gene expression by inhibiting the transcriptional regulatory functions of NF κ B. *J. Biol. Chem.* **278**: 28181–28192.
 41. Nie, M., L. Corbett, A. Knox, and L. Pang. 2005. Differential regulation of chemokine expression by peroxisome proliferator-activated receptor gamma agonists: interactions with glucocorticoids and beta2-agonists. *J. Biol. Chem.* **280**: 2550–2561.
 42. Liao, W., M. Nguyen, T. Yoshizaki, S. Favellyukis, D. Patsouris, T. Imamura, I. M. Verma, and J. M. Olefsky. 2007. Suppression of PPAR γ attenuates insulin-stimulated glucose uptake by affecting both GLUT1 and GLUT4 in 3T3-L1 adipocytes. *Am. J. Physiol. Endocrinol. Metab.* **293**: E219–E227.
 43. Pascual, G., A. Fong, S. Ogawa, A. Gamliel, A. C. Li, V. Perissi, D. W. Rose, T. M. Willson, M. G. Rosenfeld, and C. K. Glass. 2005. A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR γ . *Nature.* **437**: 759–763.
 44. Ricote, M., and C. Glass. 2007. PPARs and molecular mechanisms of transrepression. *Biochim. Biophys. Acta.* **1771**: 926–935.
 45. Straus, D., and C. Glass. 2007. Anti-inflammatory actions of PPAR ligands: new insights on cellular and molecular mechanisms. *Trends Immunol.* **28**: 551–558.
 46. Ghisletti, S., W. Huang, S. Ogawa, G. Pascual, M. E. Lin, T. M. Wilson, M. G. Rosenfeld, and C. K. Glass. 2007. Parallel SUMOylation-dependent pathways mediate gene- and signal-specific transrepression by LXRs and PPAR γ . *Mol. Cell.* **25**: 57–70.
 47. Qiao, L., and J. Shao. 2006. SIRT1 regulates adiponectin gene expression through Foxo1-C/enhancer-binding protein alpha transcriptional complex. *J. Biol. Chem.* **281**: 39915–39924.
 48. Picard, F., M. Kurtev, N. Chung, A. Topark-Ngarm, T. Senawong, R. Machado De Oliveira, M. Leid, M. W. McBurney, and L. Guarente. 2004. SIRT1 promotes fat mobilization in white adipocytes by repressing PPAR γ . *Nature.* **429**: 771–776.