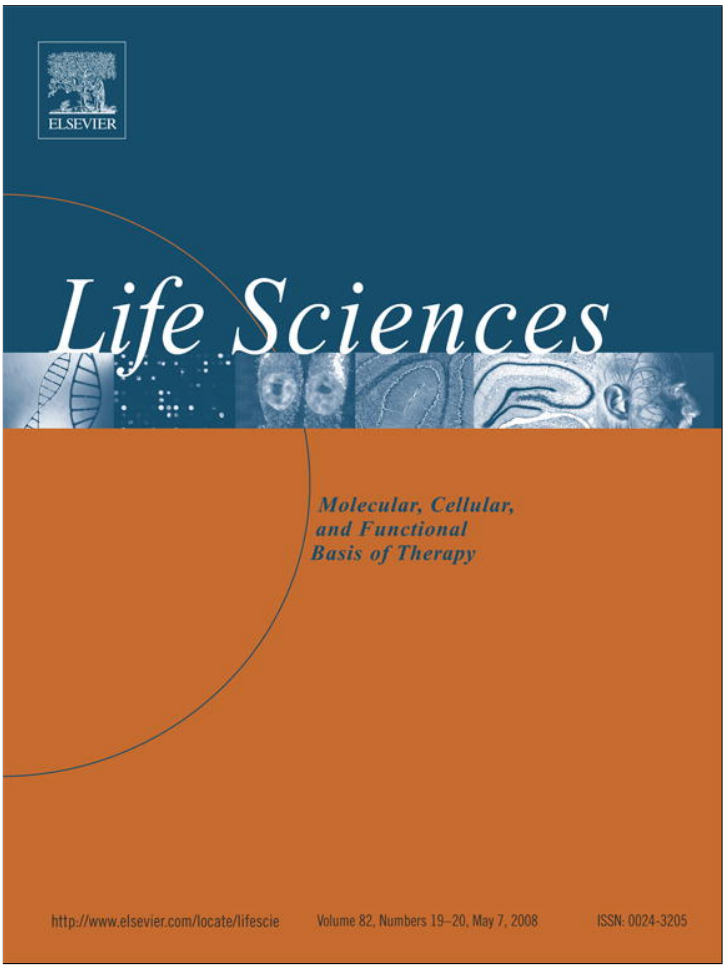

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Enhanced inhibition of adipogenesis and induction of apoptosis in 3T3-L1 adipocytes with combinations of resveratrol and quercetin

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ABSTRACT

Certain flavonoids have been shown to have specific effects on biochemical and metabolic functions of adipocytes. In this study, we investigated the effects of combinations of resveratrol and quercetin on adipogenesis and apoptosis in 3T3-L1 cells. In maturing preadipocytes resveratrol and quercetin at 25 μM individually suppressed intracellular lipid accumulation by 9.4±3.9% ($p<0.01$) and 15.9±2.5%, respectively, ($p<0.001$). The combination of resveratrol and quercetin at the same dose, however, decreased lipid accumulation by 68.6±0.7% ($p<0.001$). In addition, combinations of resveratrol and quercetin at 25 μM significantly decreased the expression of peroxisome proliferators-activated receptor gamma (PPARγ) and CCAAT/enhancer-binding protein (C/EBP)α, both of which act as key transcription factors. In mature adipocytes resveratrol and quercetin at 100 μM individually decreased viability by 18.1±0.6% ($p<0.001$) and 15.8±1% ($p<0.001$) and increased apoptosis (100 μM) by 120.5±8.3% ($p<0.001$) and 85.3±10% ($p<0.001$) at 48 h, respectively. Combinations of resveratrol and quercetin further decreased viability (73.5±0.9%, $p<0.001$) and increased apoptosis (310.3±9.6%, $p<0.001$) more than single compounds alone. The combination of resveratrol and quercetin at 100 μM increased release of cytochrome *c* from mitochondria to cytosol and decreased ERK 1/2 phosphorylation. Taken together, our data indicate that combinations of resveratrol and quercetin can exert potential anti-obesity effects by inhibiting differentiation of preadipocytes and inducing apoptosis of mature adipocytes.

Introduction

Obesity is a serious health problem because it is implicated in various diseases including type II diabetes, hypertension, coronary heart disease, and cancer (Kopelman, 2000). Obesity is characterized by increased adipose tissue mass that results from both increased fat cell number and increased fat cell size (Couillard et al., 2000). The amount of adipose tissue mass can be regulated by the inhibition of adipogenesis from fibroblastic preadipocytes to mature adipocytes (Roncari et al., 1981) and induction of apoptosis (Sorisky et al., 2000) in adipose tissues. Phytochemicals found in fruits and vegetables have potential for inhibiting adipogenesis and inducing apoptosis (Lin et al., 2005; Yang et al., 2006c), as well as prevention of cancer (Aggarwal and Shishodia, 2006; Martin, 2006). Resveratrol (R) (3,5,4′-trihydroxystilbene), a naturally occurring phytoalexin found in red wines and grape juice, has been reported to have a variety of pharmacological effects, including anti-cancer, anti-inflammatory and anti-platelet properties (Chung et al., 1992; Jang et al., 1999; Jang et al., 1997). It has also been shown to reduce the synthesis of lipids in rat liver (Arichi et al., 1982). Picard et al. showed that resveratrol activated the expression of Sirt1, and this, in turn, resulted in decreased lipid accumulation by repression of PPARγ in differentiated adipocytes (Picard et al., 2004). Resveratrol also has been shown to decrease proliferation and induce apoptosis and cell cycle arrest in various cell lines (Ferry-Dumazet et al., 2002; Haider et al., 2003; Liang et al., 2003).

Quercetin is a plant-derived, dietary flavonoid with potentially beneficial effects on cardiovascular diseases (Arts and Hollman, 2005; Hertog et al., 1993). It has been shown to inhibit glucose uptake in isolated rat adipocytes (Strobel et al., 2005) and to increase lipolysis, an effect that was synergistic with epinephrine (Kuppusamy and Das, 1992). Quercetin also has been shown to reduce cell proliferation, cause cell cycle arrest (Yoshida et al., 1992) and apoptosis in *in vitro* experiments with various cell lines (Liesveld et al., 2003; Wang et al., 1999) and in 3T3-L1 preadipocytes (Hsu and Yen, 2006).

There has been increasing interest in Western medicine in the phenomenon of enhancement of effects of herbal compounds when tested in combinations, although this phenomenon is fundamental to

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traditional systems of herbal medicine. Combinations of some compounds may synergistically increase or decrease the therapeutic activity or toxicity of drugs. Mouria et al. demonstrated that resveratrol and quercetin additively activated caspase 3 in human pancreatic carcinoma cells (Mouria et al., 2002), while Mertens-Talcott and Percival found that in combination, they synergistically induced apoptosis in human leukemia cells (Mertens-Talcott and Percival, 2005). Based on these findings, the current study was carried out to determine whether the combination of these two compounds would cause an enhanced inhibition of adipogenesis and induction of apoptosis compared to either compound by itself. We showed that in mature adipocytes, the combination of resveratrol and quercetin caused an enhanced increase in apoptosis compared to a predicted additive response, and in maturing preadipocytes the combination caused an enhanced inhibition of adipogenesis compared to the predicted additive response.

Materials and methods

Cell culture

3T3-L1 mouse embryo fibroblasts were obtained from American Type Culture Collection (Manassas, VA) and cultured as described elsewhere (Hemati et al., 1997). Briefly, cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Grand Island, NY) containing 10% bovine calf serum (BCS) until confluent. Two days after confluence (D0), the cells were stimulated to differentiate with DMEM containing 10% fetal bovine serum (FBS), 167 nM insulin, 0.5 μM isobutylmethylxanthine (IBMX), and 1 μM dexamethasone for two days (D2). Cells were then maintained in 10% FBS/DMEM medium with 167 nM insulin for another two days (D4), followed by culturing with 10% FBS/DMEM medium for an additional 4 days (D8), at which time more than 90% of cells were mature adipocytes with accumulated fat droplets. All media contained 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 292 μg/mL glutamine (Invitrogen, Carlsbad, CA). Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

Reagents and antibodies

Phosphate-buffered saline (PBS) and DMEM medium were purchased from GIBCO (BRL Life Technologies, Grand Island, NY). Trans-resveratrol (trans-3,5,4′-trihydroxystilbene, purity>99.0%; R), quercetin (3′,4′,5,7-tetrahydroxyflavan-3-ol, purity>98.0%; Q), and bis-benzimide H 33342 were purchased from Sigma (St. Louis, MO). ApoStrand™ ELISA Apoptosis Detection Kit was purchased from BIOMOL (Plymouth Meeting, PA). The viability assay kit (CellTiter 96 Aqueous One Solution Cell Proliferation Assay) was purchased from Promega (Madison, WI). AdipoRed assay reagent was purchased from Cambrex, Bio Science Walkersville, Inc. (Walkersville, MD). Antibodies specific for polyclonal PPARγ2, C/EBPα, β-Actin, and cytochrome *c* were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies specific for polyclonal anti-phospho-ERK1/2 (Thr^{202}/Tyr^{204}) and total ERK1/2 were from Cell Signaling Technology (Beverly, MA).

MTS cell viability assay

Tests were performed in 96-well plates. For mature adipocytes, cells were seeded (5000 cells/well) and grown to maturation as described above. Mature adipocytes were incubated with DMSO or test compounds for 24 and 48 h. Cell viability was monitored by the Cell Proliferation MTS Kit as recommended by the manufacturer (Woods et al., 2008). Prior to measuring viability, treatment media were removed and replaced with 100 μl fresh 10% FBS/DMEM medium and 20 μl 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) solution. Cells were then returned to the incubator for an additional two hours before 25 μl of 10% SDS was added to stop the reaction. The absorbance was measured at 490 nm in a plate reader (μQuant™ Bio-Tek Instruments, Inc., Winooski, VT) to determine the formazan concentration, which is proportional to the number of live cells.

Apoptosis assays

For the assessment of apoptosis, the ApoStrand™ ELISA Apoptosis Detection Kit (Biomol, Plymouth Meeting, PA) was used. This kit detects single stranded DNA, which occurs in apoptotic cells but not in necrotic cells or in cells with DNA breaks in the absence of apoptosis (Frankfurt, 2004; Frankfurt and Krishan, 2001). Tests were performed in 96-well plates. For mature adipocytes, cells were seeded (5000 cells/well) and grown to maturation as described above. Prior to ssDNA ELISA, adipocytes were incubated with either DMSO or test reagents for 24 and 48 h. Thereafter, treatment medium was removed and the cells were fixed for 30 min and assayed according to the manufacturer's instructions.

Hoechst staining

The cells were seeded on culture dishes, grown to confluence, and induced to differentiate. After the indicated treatment, cells were processed for Hoechst staining (H-33342; Sigma, St Louis, MO). Briefly, the cells were fixed in 10% formaldehyde, washed with PBS, and stained with Hoechst dye, followed by extensive washes. Nuclear staining was examined under a fluorescence microscope (100× magnification) and three images for each dish were captured using ImagePro software version 5.1 (MediaCybernetics, Silver spring, MD).

Oil Red O staining

Test compounds along with 0.2% DMSO control were added with the induction medium for days 0–6 of adipogenesis. Medium was changed every two days. On day 6, cells were stained with Oil Red O. In brief, cell monolayers were washed twice with PBS and fixed with 10% formalin in PBS (pH 7.4): cells were then stained with Oil Red O and hematoxylin as described by Suryawan and Hu (Suryawan and Hu, 1993). After mounting with glycerol gelatin, three images for each dish were captured using ImagePro software (MediaCybernetics, Silver spring, MD).

Quantification of lipid content by AdipoRed™ assay

Lipid content was measured using a commercially available kit (AdipoRed Assay Reagent). AdipoRed, a solution of the hydrophilic stain Nile Red, is a reagent that enables the quantification of intracellular lipid droplets in a high-throughput manner. In brief, for the experiment shown in Fig. 1A, test compounds along with 0.2% DMSO control were added with the induction medium for days 0–6 of adipogenesis. Medium was changed every two days. On day 6, intracellular lipid content was measured by AdipoRed Assay. Cells were washed with PBS (pH 7.4) and 200 μL of PBS was added to the wells. AdipoRed reagent (5 μL) was added to each well. After 10 min, the plates were placed in the fluorimeter and fluorescence was measured with excitation wavelength of 485 nm and emission wavelength of 572 nm.

Western blot analysis

To examine the effect of test compounds on expressions of PPARγ2, C/EBPα and C/EBPβ, the differentiation of 2-day post-confluent 3T3-L1 preadipocytes was induced by standard adipogenic medium as described above. Test compounds were added during days 0–6 with the standard adipogenic medium. On day 6, cells were washed with PBS

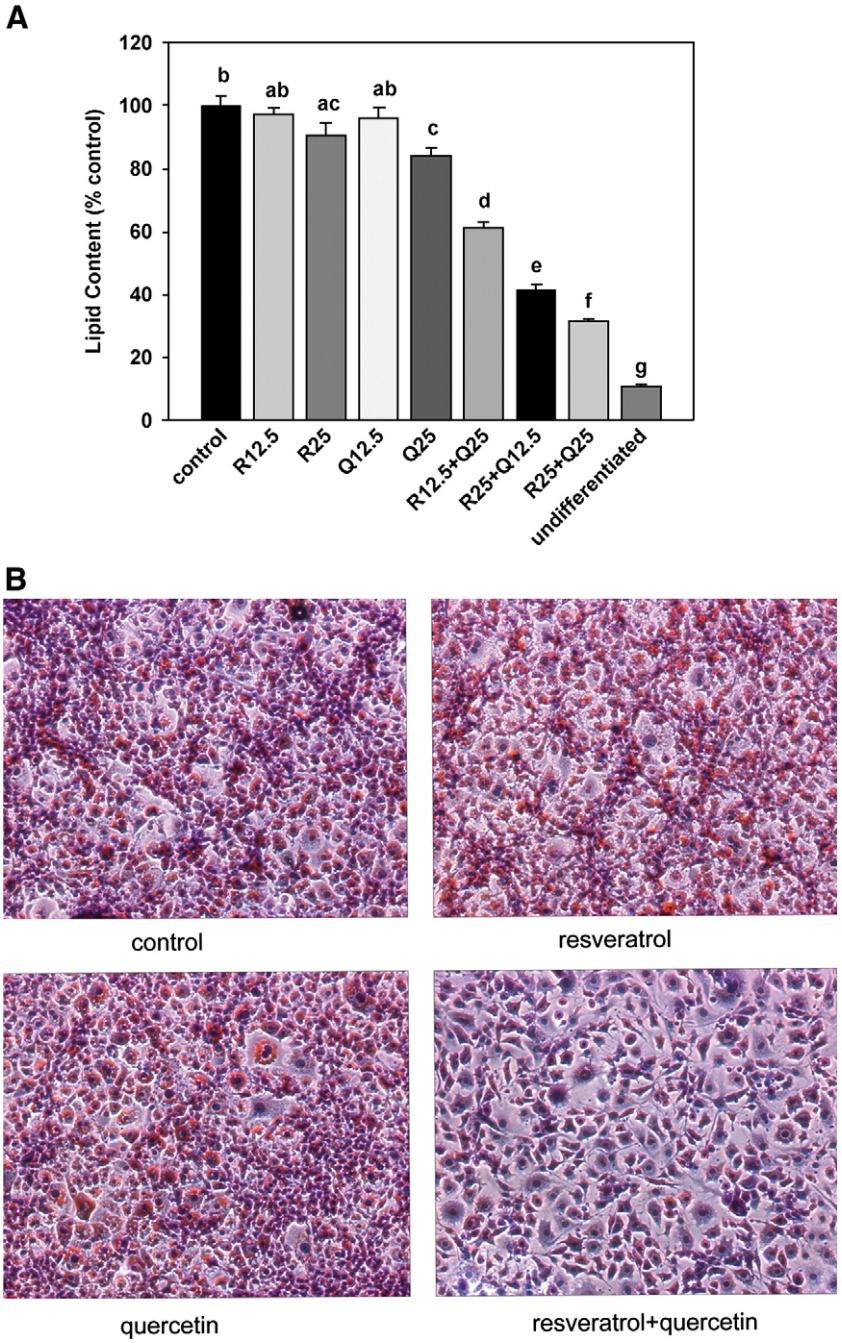

Fig. 1. Resveratrol and quercetin inhibit lipid accumulation during adipocyte differentiation. The differentiation of 2-day post-confluent 3T3-L1 preadipocytes was induced by standard adipogenic medium to initiate adipogenesis as described in "Materials and methods". (A) Resveratrol (R; 12.5, 25 μM), quercetin (Q; 12.5, 25 μM), and resveratrol plus quercetin (R+Q; 12.5+25 μM, 25+12.5 μM, 25+25 μM) were added during days 0–6 with the standard adipogenic medium. On day 6, lipid content was measured by AdipoRed assay. All assays were performed on eight replicates for each treatment. Means that are not denoted with a common letter are different, abcdefg: $p<0.05$. (B) Resveratrol (25 μM), quercetin (25 μM), and resveratrol plus quercetin (25+25 μM) were added during days 0–6 with the standard adipogenic medium. On day 6, cellular triglyceride was stained with Oil red O and photographed at 100× magnification. The experiment was performed in triplicate.

and suspended in a lysis buffer (20 mM Tris, pH 7.5, 150 mM sodium chloride, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium vanadate (Na_3VO_4), 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 100 μg/mL phenylmethylsulfonyl fluoride (PMSF). To examine the effect of test compounds on expression of ERK1/2 phosphorylation, mature 3T3-L1 adipocytes were treated with either DMSO or test compounds for 30 min. After incubation, cells were washed with ice-cold PBS and suspended in a lysis buffer as described above. After 30 min of rocking at 4 °C, the mixtures were centrifuged (10,000 ×*g*) for 10 min, and the supernatants were collected as the whole-cell extracts.

To examine the effect of test compounds on the expression of cytochrome *c*, mature 3T3-L1 adipocytes were treated with either DMSO or test compounds for 48 h. After incubation, cells were washed with ice-cold PBS and resuspended in isotonic homogenizing buffer (250 mM sucrose, 10 mM potassium chloride, 1.5 mM magnesium chloride, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 10 mM HEPES-KOH, pH 7.4). After 30 min incubation on ice, cells were homogenized with a glass Dounce homogenizer (30 strokes) and centrifuged at 700 ×*g* for 10 min, then 10,000 ×*g* for 20 min, and finally 100,000 ×*g* for another 60 min at 4 °C for the collection of the cytosolic fraction. The

supernatant was collected as the cytosolic fraction and the resulting mitochondrial pellets were dissolved in lysis buffer described previously. The protein concentration was determined by the method of Bradford (Bradford, 1976) with bovine serum albumin as the standard. Western blot analysis was performed using the commercial NuPAGE system (Novex/Invitrogen, Carlsbad, CA), where a lithium dodecyl sulfate (LDS) sample buffer (Tris/glycerol buffer, pH 8.5) was mixed with fresh dithiothreitol and added to samples. Samples were then heated to 70 °C for 10 min. All cell lysates were separated by 12% acrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline and then incubated with primary antibodies overnight at 4 °C. After thorough washing, the membranes were incubated with secondary antibodies conjugated with horseradish peroxidase for 1 h at room temperature. Protein signals were developed using the ECL system from Amersham Biosciences (Piscataway, NJ, U.S.A.). All experiments were repeated at least two times. Representative Western blots are shown along with the graphs of the quantitative data.

Quantitative analysis of Western blot data

Measurement of signal intensity on PVDF membranes after Western blotting with various antibodies was performed using a FluorChem™densitomer with the AlphaEaseFC™ image processing and analysis software (Alpha Innotech Corporation). For statistical analysis, all data were expressed as integrated density values (IDV). For PPARγ and C/EBPα, the IDVs were calculated as the density values of the specific protein bands/β-actin density values and expressed as percentage of the control. For phospho-p44 ERK and phospho-p42 ERK, IDVs were calculated as the density values of the specific protein bands/total ERK density values and expressed as percentage of the control. For cytochrome *c*, IDVs were calculated as the density values of the specific protein bands/β-actin density values and expressed as percentage of the control. All figures showing quantitative analysis include data from at least three independent experiments.

Statistical analysis

One-way analysis of variance (GLM procedure, Statistica, version 6.1; StatSoft, Inc.) was used to determine significance of treatment effects. In two experiments (lipid accumulation in maturing preadipocytes and viability in mature adipocytes) in order to estimate differences between the combination treatments and a hypothetical additive treatment response, a sum of the individual treatment effects for each replicate was calculated and these numbers were included in the ANOVA (for example, in the cell viability assay, the absorbance values obtained upon treatment with resveratrol alone were added to the absorbance values obtained with quercetin treatment (RQ-calculated). These numbers were included in the statistical analysis along with the absorbance values obtained after treating the cells with R and Q in combination, (R+Q-combined). Fisher's post-hoc least significant difference test was used to determine significance of

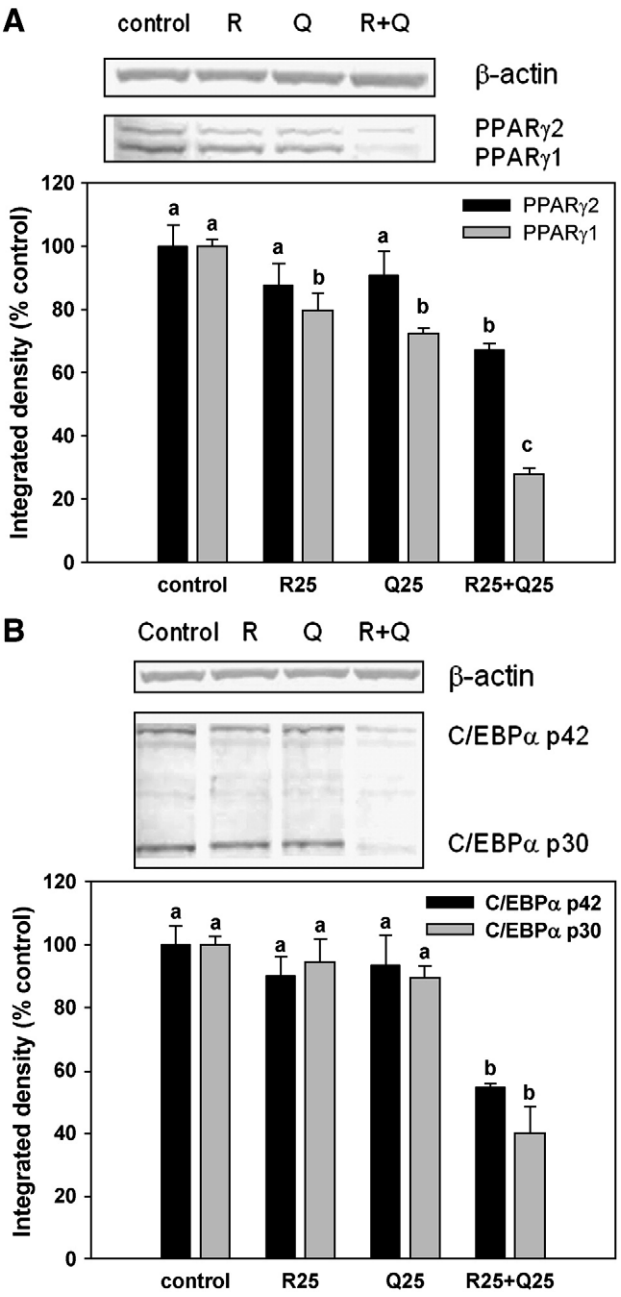

Fig. 2. Resveratrol and quercetin decrease PPARγ and C/EBPα expression. The differentiation of 2-day post-confluent 3T3-L1 preadipocytes was induced by standard adipogenic medium to initiate adipogenesis as described in "Materials and Methods" in the absence or presence of 25 μM resveratrol, 25 μM quercetin, and resveratrol plus quercetin (25+25 μM). On day 6, cells were lysed, separated on 10% NuPAGE and subjected to immunoblotting to examine the expression levels of PPARγ (2A) and C/EBPα (2B). Actin was used as an internal reference for sample loading control. Densitometric quantitation of the autoradiograms for PPARγ and C/EBPα was performed. Integrated density values were calculated and expressed as % control. All experiments were performed in triplicate. Means that are not denoted with a common letter are different (abc: $p<0.05$).

Table 1
Percent decrease in lipid accumulation in maturing preadipocytes treated with resveratrol (R), quercetin (Q) and the combined treatments for 6 days

Treatment	% Decrease in lipid accumulation (Mean±SEM)
Control	0.0 ± 3.1^{u}
R12.5	2.9 ± 2.2^{uv}
R25	9.4 ± 3.9^{uvw}
Q12.5	4.1 ± 3.6^{uv}
Q25	15.9 ± 2.5^{vwx}
R12.5Q25 (calculated)*	18.8 ± 3.8^{wx}
R25Q25 (calculated)*	25.4 ± 5.4^{xy}
R25Q12.5 (calculated)*	13.6 ± 6.8^{uvwx}
R12.5+Q25 (combined)	38.9 ± 1.8^{y}
R25+Q12.5 (combined)	58.6 ± 1.7^{z}
R25+Q25 (combined)	68.6 ± 0.7^{z}

uvwxyz: means not denoted with a common letter are different, $p<0.01$.
* Calculated additive response is the sum of the effects of the individual R and Q treatments.

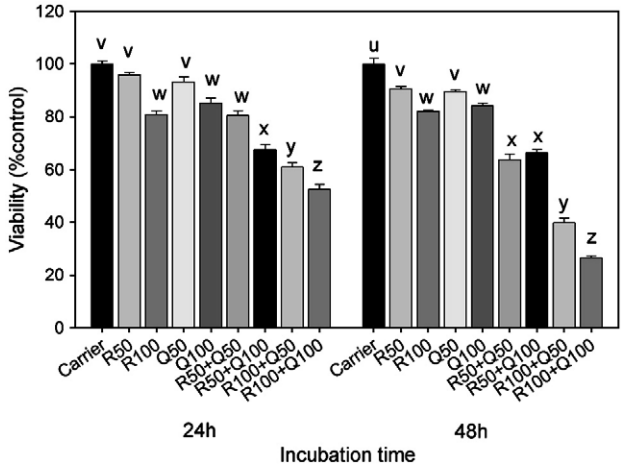

Fig. 3. Resveratrol and quercetin reduce cell viability in mature adipocytes. Mature 3T3-L1 adipocytes were incubated with resveratrol (R; 50, 100 μM), quercetin (Q; 50, 100 μM), and resveratrol plus quercetin (R+Q; 50+50 μM, 50+100 μM, 100+50 μM, 100+100 μM) for 24 and 48 h. Cell viability was determined by the MTS colorimetric assay. Assays were performed on eight replicates for each treatment. Within a time period means that are not denoted with a common letter are different, uvwxyz: $p<0.01$.

differences among means. Statistically significant differences are defined at the 95% confidence interval. Data shown are means ± SEM.

Results

Effect of resveratrol and quercetin on intracellular lipid accumulation during adipocyte differentiation

During the differentiation of 3T3-L1 preadipocytes to adipocytes, the cells were treated with R and Q as individual compounds and in combination during day 0–6, and intracellular lipid content was measured and quantified. R and Q at 25 μM each suppressed lipid accumulation by 9.4±3.9% ($p<0.01$) and 15.9±2.5% ($p<0.001$) after 6 days of treatment, respectively, but did not affect viability (data not shown). The combination of R and Q (R+Q) at 25 μM each further decreased lipid accumulation by 68.6±0.7% ($p<0.001$) (Fig. 1A). Analysis of the calculated additive decrease in lipid accumulation with R and Q showed that the combined treatments caused significantly greater decrease in lipid accumulation compared to additive decreases with R and Q (Table 1). For subsequent adipogenesis experiments, R and Q were tested at 25 μM.

Table 2
Percent decrease in viability of mature adipocytes treated with resveratrol (R), quercetin (Q) and the combined treatments after 48 h treatment

Treatment	% Decrease in viability
	Mean ± SEM
Control	0.0 ± 2.0^{s}
R50	9.8 ± 1.0^{t}
R100	18.1 ± 0.6^{u}
Q50	10.7 ± 0.9^{t}
Q100	15.8 ± 1.0^{u}
R50Q50 (calculated)*	20.5 ± 1.1^{u}
R100Q50 (calculated)*	28.8 ± 1.2^{vw}
R50Q100 (calculated)*	25.6 ± 1.8^{v}
R100Q100 (calculated)*	34.0 ± 1.1^{x}
R50+Q50 (combined)	36.3 ± 2.1^{x}
R50+Q100 (combined)	33.6 ± 1.2^{wx}
R100+Q50 (combined)	60.2 ± 1.8^{y}
R100+Q100 (combined)	73.5 ± 0.9^{z}

stuvwxyz: means not denoted with a common letter are different, $p<0.01$.
* Calculated additive response is the sum of the effects of the individual R and Q treatments.

Similar results were observed using Oil Red O staining to visualize lipid accumulation in cells after treatments. As shown in Fig. 1B, in the group treated with 25 μM resveratrol and quercetin alone, cells were rounded with increasing lipid, similar to control, while in the group treated with R+Q, cells retained some of the fibroblastic characteristics and some cells were rounded with decreased amounts of lipid.

Effect of resveratrol and quercetin on expression of PPARγ and C/EBPα

To investigate the inhibitory mechanism of R and Q during the differentiation period (0–6 day), the expression of PPARγ and C/EBPα as transcriptional factors for adipocyte differentiation were examined. R and Q at 25 μM decreased the expression of PPARγ1 by 20.3±5.3% ($p<0.01$) and 27.6±1.4% ($p<0.001$), respectively, whereas R and Q had no significant effect on the expression of PPARγ2 (Fig. 2A) as individual compounds. Combination of R+Q at 25 μM each significantly decreased the expression of PPARγ1 by 72.2±1.9% ($p<0.001$) and PPARγ2 by 32.9±1.9% ($p<0.001$). Neither R nor Q at 25 μM had an effect on the expression of C/EBPα p42 or p30, whereas R+Q significantly decreased the expression of C/EBPα p42 by 45.4±1.2% ($p<0.001$) and p30 by 60.2±8.4% ($p<0.001$) (Fig. 2B).

Effect of resveratrol and quercetin on viability of mature adipocytes

3T3-L1 mature adipocytes were treated with R and Q as individual compounds and in combinations at various doses for 6, 12, 24, and 48 h. Data from 24 and 48 h are shown in Fig. 3. After treatment, the number of live cells was determined by the MTS assay. R and Q individually decreased viability at 50 and 100 μM. R+Q (50, 100 μM each) further decreased viability more than the additive responses to individual compound at 48 h, and the calculated additive responses were significantly less than the corresponding combined treatments (Table 2).

Effect of resveratrol and quercetin on apoptosis of mature adipocytes

We next investigated whether the reduction in cell number by resveratrol and quercetin was due to apoptosis. 3T3-L1 mature adipocytes were treated with R and Q as individual compounds and in combinations at various doses for 24 and 48 h. After treatment, apoptosis was measured by the ssDNA ELISA assay. As shown in Fig. 4, R and Q at 100 μM increased apoptosis by 120.5±8.3% ($p<0.001$) and

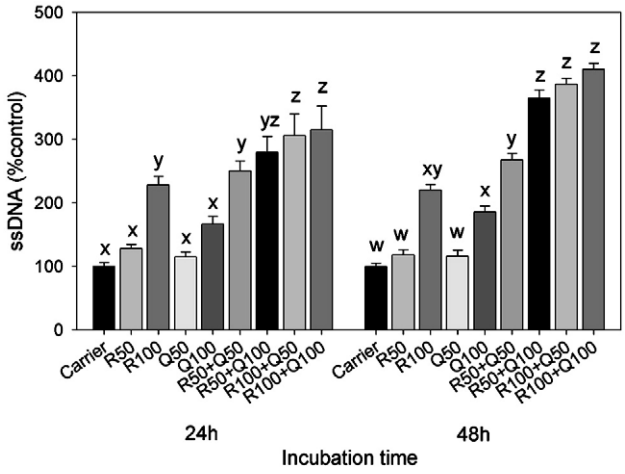

Fig. 4. Resveratrol and quercetin induce apoptosis in mature adipocytes. Mature 3T3-L1 adipocytes were incubated with resveratrol (R; 50, 100 μM), quercetin (Q; 50, 100 μM), and resveratrol plus quercetin (R+Q; 50+50 μM, 50+100 μM, 100+50 μM, 100+100 μM) for 24 and 48 h. Cell apoptosis was evaluated by ssDNA ELISA. Assays were performed on eight replicates for each treatment. Within a time period means that are not denoted with a common letter are different, wxyz: $p<0.05$.

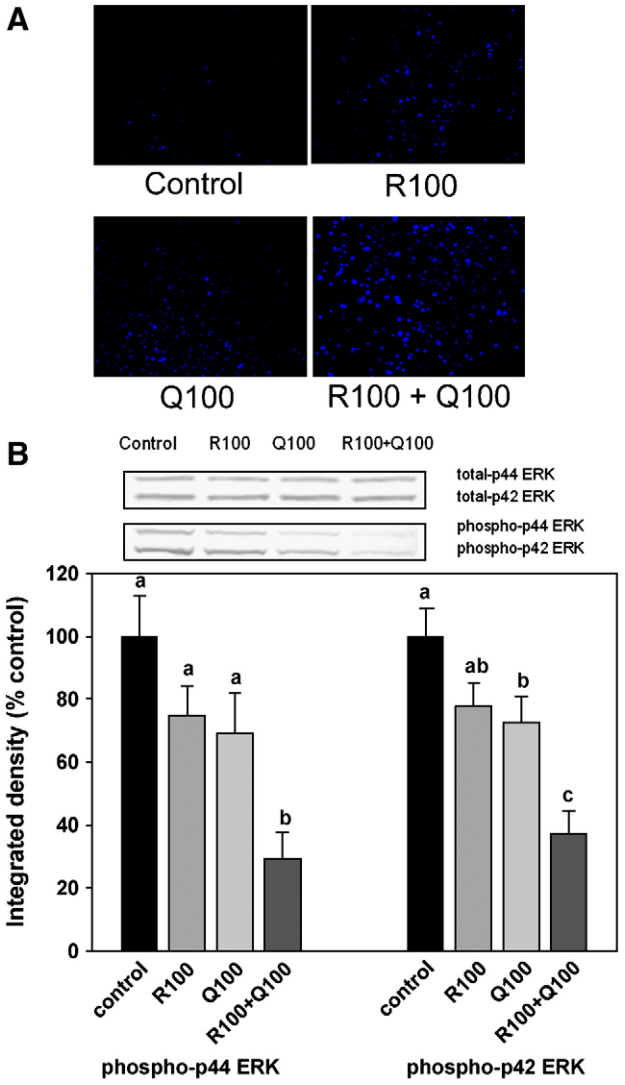

Fig. 5. Fluorescence microscopy shows apoptosis and ERK modulation by resveratrol and quercetin in mature adipocytes. (A) Mature 3T3-L1 adipocytes were incubated with resveratrol (100 μM), quercetin (100 μM), and resveratrol plus quercetin (100+100 μM) for 24 h and cells were stained with H-33342 and mounted onto glass slides as a wet preparation. The slides were photographed under fluorescence microscopy at 200× magnification. The experiment was performed in triplicate. (B) Mature 3T3-L1 adipocytes were incubated with resveratrol (100 μM), quercetin (100 μM), and resveratrol plus quercetin (100+100 μM) for 30 min. The protein levels of total and phosphorylated forms of ERK 1/2 were evaluated in cytosolic proteins by Western blotting with the use of specific antibody. Representative Western blots show the phosphorylation of ERK 1/2 in the lower panel and the expression levels of the total kinase in the upper panel. All experiments were repeated three times. Means that are not denoted with a common letter are different, abc: $p<0.05$.

85.3±10% ($p<0.001$), respectively, after 48 h of treatment, while individual compounds at 50 μM had no significant effect. The combination of R+Q increased apoptosis in a time- and dose-dependent manner. R+Q (100 μM each) further increased apoptosis 215.1±37.5% ($p<0.001$) and 310.3±9.6% ($p<0.001$) more than control after 24 and 48 h incubation periods respectively. The calculated additive increase in apoptosis for R 100 μM+Q 100 μM at 48 h was 205.8±14.8%, which is less ($p<0.001$) than the combined treatment of the same concentrations.

Effect of resveratrol and quercetin on morphological change of apoptosis and on ERK activation

The enhanced effect of R+Q (100 μM) on apoptosis was further confirmed using fluorescence microscopy of cells treated with Hoechst stain (Fig. 5A), which is based on the observation that the nucleus of apoptotic cells stains with a higher-intensity than that of normal cells (Ormerod et al., 1993). Normal nuclei stained with H-33342 fluoresce faintly, and a slight increase in fluorescence as compared to control cells was observed with cells exposed to R or Q for 24 h. The fluorescent intensity of cells treated with R+Q was greater than the responses to resveratrol or quercetin alone. The increased intensity under these conditions is believed to be both the result of chromatin condensation as well as an increased rate of influx of H-33342 into apoptotic cells.

Several studies have shown that the ERK pathway is involved in cell survival, cell cycle progression and apoptosis (Chang et al., 2003; Fan and Chambers, 2001). Therefore, we tested whether ERK activation was involved in mediating the effect of resveratrol and quercetin-induced apoptosis. R alone decreased ERK phosphorylation (both phospho-p42 ERK and phospho-p44 ERK) by about 24%, although the decrease wasn't significant. Q alone also decreased ERK phosphorylation (p44 ERK phosphorylation by 30.9±12.8% and p42 ERK phosphorylation by 27.6±8.5%, $p<0.05$). The combination of resveratrol+quercetin decreased ERK phosphorylation significantly more than resveratrol or quercetin alone (Fig. 5B) (phospho-p44 ERK: 70.6±8.2%, $p<0.001$, and phospho-p42 ERK: 62.7±7.1%, $p<0.001$).

Effect of resveratrol and quercetin on cytosolic cytochrome c release

Since one of the major apoptotic pathways is triggered by the release of mitochondrial cytochrome *c*, we also examined whether the enhanced increase in apoptosis by R+Q involved cytochrome *c*. As shown in Fig. 6, R (100 μM) induced cytochrome *c* release by 74.6±23.2% ($p<0.05$), whereas Q (100 μM) alone caused a small, but not significant increase. R+Q increased cytochrome *c* release by 349.5±20.1% ($p<0.001$).

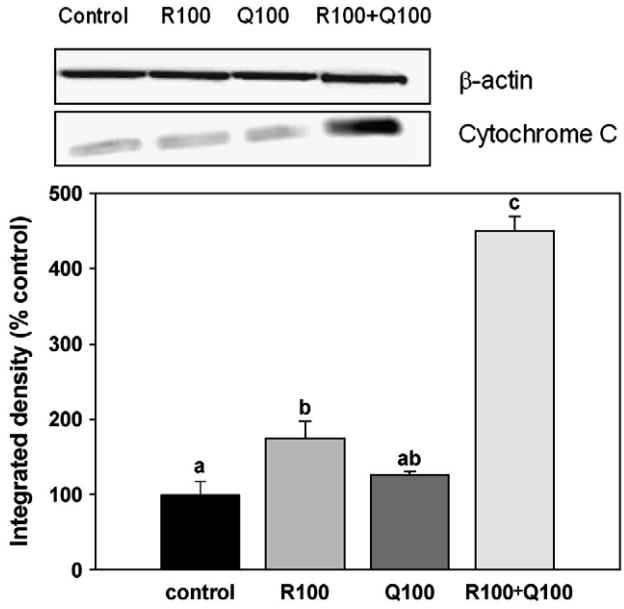

Fig. 6. Resveratrol and quercetin induce cytochrome *c* release in mature adipocytes. Mature 3T3-L1 adipocytes were incubated with resveratrol (100 μM), quercetin (100 μM), and resveratrol plus quercetin (100+100 μM) for 48 h. Cytochrome *c* protein from cytosolic fraction was analyzed by Western blotting using an anti-cytochrome *c* antibody. β-actin was used as an equal loading control. Densitometric quantitation of the autoradiogram was performed. Integrated density values (IDV) (cytochrome *c*/actin) were calculated and expressed as % control. All experiments were repeated three times. Means that are not denoted with a common letter are different, abc: $p<0.05$.

Discussion

In this study we found that combination treatment with resveratrol and quercetin suppressed lipid accumulation significantly more than the responses to resveratrol or quercetin alone and more than the calculated additive response during the differentiation period in 3T3-L1 cells. Moreover, the adipocyte-specific transcription factors PPARγ and C/EBPα were decreased by combination of resveratrol and quercetin, whereas at the tested concentrations, the compounds by themselves either had no effect or were much less effective than in combination. PPARγ and C/EBPα play important roles during adipocyte differentiation because they are transcriptional factors for numerous genes (Gregoire et al., 1998). Several studies have demonstrated that PPARγ2 and C/EBPα co-regulate each other's expression. PPARγ2 is the ultimate key regulator of adipogenesis, and C/EBPα might play more of an accessory role for PPARγ2 by inducing and maintaining PPARγ2 expression (Camp et al., 2002).

In a transient reporter assay some flavonoids, such as apigenin and kaempferol, were able to activate PPARγ, and the activation of PPARγ was apparently dependent on the number and the position of hydroxyl residues (Liang et al., 2001). However, some flavonoids, such as luteolin and quercetin, decreased the activation of PPARγ in RAW264.7 cells. A recent study also suggests that resveratrol-induced activation of Sirt1 is responsible for fat mobilization in mature adipocytes and activation of Sirt1 also decreased the expression of PPARγ, resulting in decreased fat accumulation (Picard et al., 2004). Consistent with these results, our study showed that the combination of resveratrol and quercetin caused an enhanced decrease in the expression of PPARγ during the differentiation period (0–6 day) at concentrations as low as 25 μM.

The amount of adipose tissue mass can be decreased by deletion of adipocytes via apoptosis as well as by inhibition of adipogenesis. Therefore, we investigated whether resveratrol and quercetin had apoptotic activity in mature adipocytes. Resveratrol and quercetin individually decreased viability and combinations of resveratrol and quercetin further decreased viability more than the additive responses at 48 h. We further showed that the decrease in viability was at least partly due to an increase in apoptosis. Combinations of resveratrol and quercetin increased apoptosis in a time- and dose-dependent manner. Possible mechanisms of action for the inhibition of viability and increase of apoptosis with the combination of resveratrol and quercetin could involve mitogen-activated protein (MAP) kinases, especially the extracellular signal-regulated kinases (ERKs). The ERK pathway is mainly responsive to mitogens and growth factors and plays a key role in cell proliferation, survival, and differentiation (Cobb, 1999). Moreover, in a study of several tumor cell lines, including those derived from breast, lung, and ovary carcinomas, inhibition of ERK signaling enhanced apoptosis (MacKeigan et al., 2000). Consistent with these results, our study showed that the combination of resveratrol and quercetin decreased the phospho-ERK1/2 levels more than the responses to the individual compounds.

Cytochrome *c* release from mitochondria to the cytosol is also known to mediate apoptosis. Cytochrome *c* release is regulated by several Bcl-2 family proteins (Adams and Cory, 1998; Yang et al., 2006b). Recent studies have shown that resveratrol (Sareen et al., 2006) and quercetin (Yang et al., 2006a) induced apoptosis and release of cytochrome *c* in cancer cells. Interestingly, in our study the expression pattern for the release of cytochrome *c* was different for resveratrol and quercetin. In resveratrol-treated cells, cytochrome *c* level was higher than in quercetin-treated cells. The combination of resveratrol and quercetin increased cytochrome *c* more than the calculated additive responses to individual compounds at 48 h. These results are consistent with apoptosis induction. Although both of these flavonoids were effective by themselves in inducing apoptosis, we showed that in combination they exert more potent effects than as individual compounds. Similar potentiated effects have been reported with genistein plus resveratrol and ajoene plus CLA (Rayalam et al., 2007; Yang et al., 2007), indicating that combination of natural compounds is more potent than the individual compounds in inhibiting adipogenesis and inducing apoptosis in adipocytes.

In summary, we showed that the combination of resveratrol and quercetin attenuated lipid accumulation through down-regulation of PPARγ and C/EBPα and induced apoptosis through suppression of ERK1/2 phosphorylation and activation of the mitochondria pathway. These findings provide information about the molecular mechanisms by which dietary flavonoids influence the regulation of fat cell volume and number, and further suggest that resveratrol and quercetin, especially in combination, may have a potential to be used for regulating the adipocyte life cycle.

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