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Rosemary (*Rosmarinus officinalis* L.) Extract Regulates Glucose and Lipid Metabolism by Activating AMPK and PPAR Pathways in HepG2 Cells

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ABSTRACT: An epidemic of metabolic disorders such as obesity and diabetes is rising dramatically. Using natural products as potential preventive and therapeutic interventions for these disorders has drawn worldwide attention. Rosemary has been shown to lower blood glucose and cholesterol levels and mitigate weight gain in several *in vivo* studies. However, the mechanisms are essentially unknown. We investigated the effects of rosemary extract on metabolism and demonstrated that rosemary extract significantly increased glucose consumption in HepG2 cells. The phosphorylation of AMP-activated protein kinase (AMPK) and its substrate, acetyl-CoA carboxylase (ACC), was increased by rosemary extract. Rosemary extract also transcriptionally regulated the genes involved in metabolism, including SIRT1, PPARγ coactivator 1α (PGC1α), glucose-6-phosphatase (G6Pase), ACC, and low-density lipoprotein receptor (LDLR). Furthermore, the PPARγ-specific antagonist GW9662 diminished rosemary's effects on glucose consumption. Overall, our study suggested that rosemary potentially increases liver glycolysis and fatty acid oxidation by activating AMPK and PPAR pathways.

KEYWORDS: *rosemary (Rosmarinus officinalis L.), glucose metabolism, fatty acid metabolism, AMPK, PPAR*

INTRODUCTION

Culinary herbs and spices have a long history of use to prevent and treat diseases, including type-2 diabetes mellitus (T2DM).[1−3] Rosemary (*Rosmarinus officinalis* L.) is used as a spice in traditional Mediterranean cuisine. Its extracts are natural antioxidants with reported therapeutic applications in folk medicines for a wide range of health conditions such as Alzheimer's disease,[4] cancer,[5,6] cardiovascular disease,[7,8] T2DM,[9−12] and obesity.[13−16] In a recent clinical study, oral administration of rosemary extracts for 21 days increased serum plasminogen-activator-inhibitor-1 (PAI-1) level (a biomarker of endothelial function) and improved endothelial dysfunction in young, healthy volunteers.[7] A number of animal studies demonstrated that rosemary extracts mitigate weight gain and improve plasma glucose and lipid profiles.[12−14,17] C57BL/6J mice that consumed 200 mg/kg body weight (BW) of rosemary extract showed a significant reduction of weight and fat mass gain (−64% and −57%, respectively) induced by a high-fat diet (HFD).[14] In a separate study, Ibarra et al. also demonstrated that mice supplied with 500 mg/kg BW of rosemary extract in their HFD had 69% less body weight increase than those in the HFD alone group.[13] Even more intriguing, rosemary extract also reduced fasting plasma glucose and cholesterol levels in those mice.[13] Along the same line, rosemary extract at 200 mg/kg BW significantly lowered blood glucose level in alloxan-induced diabetic rabbits.[12] Even with all these promising data, the mechanisms by which rosemary regulates lipids and glucose metabolism are not well understood. Several studies have indicated that rosemary extract or its well-known components, such as rosmarinic acid, carnosic acid, carnosol, and caffeic acid, may regulate the activity of PPARγ,[10] differentiation of adipocytes,[15] and inhibit pancreatic lipase.[13,14,18]

In the present study, we investigated the effect of rosemary extract on two critical pathways in metabolic regulation, AMP-activated protein kinase (AMPK) and peroxisome proliferated-activated receptor (PPAR), in addition to glucose consumption and glycolysis in hepatocellular carcinoma HepG2 cells. AMPK is a serine/threonine kinase that functions as an intracellular energy sensor, activated under conditions of low energy, such as elevated AMP/ATP ratio.[19] AMPK is also activated by physiological stimuli, including exercise, hormones such as adiponectin and leptin, and stress such as glucose deprivation, hypoxia, and oxidative stress.[20−22] The activation of AMPK switches off anabolic pathways that consume ATP, such as fatty acid, glycogen, and cholesterol synthesis, and switches on catabolic pathways that generate ATP, such as fatty acid oxidation and glycolysis.[22] Nuclear receptors PPARα, -γ, and -δ function as sensors for fatty acids and fatty acid derivatives and control expressions of many essential genes in the regulation of cellular metabolism (carbohydrate, lipid, protein), differentiation, and development.[23] Our data demonstrated that rosemary extract activated both AMPK and PPAR pathways, thereby regulating glucose and lipid metabolism in HepG2 cells.

MATERIALS AND METHODS

Reagents. Metformin, GW9662, antibody against β-actin, *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA), and 5-methylimidazole were purchased from Sigma (St. Louis, MO). Anti-phospho-AMPKα, anti-AMPKα, antiphospho-ACC, and anti-ACC were purchased from Cell Signaling (Danvers, MA).

Preparation of Rosemary Extracts (RE). Whole dried Albanian rosemary (*Rosmarinus officinalis* L.) was ground and passed through a

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40 mesh sieve and extracted following protocols established by the National Cancer Institute.[24] Briefly, the ground plant was steeped overnight (16 h) in dichloromethane−methanol (CH_2Cl_2−MeOH, 1:1). The filtrate was collected under slight vacuum, and then a MeOH extraction was performed for 30 min. The solvent was removed and combined with the other extract. The solvent was removed from the combined extracts by rotary evaporation. Aliquots of RE were prepared in DMSO at 10 mg/mL and stored at −20 °C for bioassay evaluation.

GC-MS Analysis of RE. RE was analyzed by GC-MS following the procedure outlined by Razborsek et al.[25] The GC-MS procedure required a 100 μL portion of the rosemary extract taken to dryness in a 4 mL vial under a stream of nitrogen. The residue was treated with 100 μL of MSTFA and 20 μL of *N*-methylimidazole and heated at 70 °C for 2 h. After cooling to room temperature the solution was diluted to 1 mL with tetrahydrofuran (THF) and analyzed by GC-MS. The GC-MS conditions were as follows: LECO Pegasus III GC-MS TOF instrument (LECO Corporation, St. Joseph, MI) equipped with a DB-5 ms column (Agilent Technologies, Santa Clara, CA), 30 m × 0.25 mm × 0.25 μ capillary, He carrier @ 40 cm/s. Oven program: 105 °C (0.8 min) to 220 °C (0 min) @ 15 °C/min, then to 300 °C (20 min) @ 40 °C/min. Injector temperature 290 °C, split ratio 1:75, 2 μL injection volume. Transfer line was set to 290 °C, source temperature 235 °C, TIC scan mode *m*/*z* 50−750 with a 20 min solvent delay. The trimethylsilyl (TMS) derivatives of each compound were identified by mass spectrometry and quantitated by comparison to TMS derivatives of rosemary analytical standards.

Cell Culture and Treatment. HepG2 cells were purchased from ATCC (HB-8065) and maintained in DMEM low glucose (Invitrogen, Grand Island, NY) supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin (Invitrogen) at 37 °C/5% CO_2. Before experiments, HepG2 cells were serum-starved for 24 h in DMEM low-glucose medium. After serum starvation, the cells were treated with or without different concentrations of RE or metformin for 4 h (2 h for Western blotting and glycolysis assay). Metformin stock solution was prepared in PBS at 100 mM, aliquotted, and stored at −20 °C. The working concentration of metformin in our studies was 5 mM.

LDH Cytotoxicity Assay. Cells were treated with different doses of RE or metformin for 4 h. Lactate dehydrogenase (LDH) released into culture supernatants was detected by colorimetric enzyme-linked immunosorbent assay, using the LDH cytotoxicity detection kit (Clontech, Mountain View, CA) according to the manufacturer's instructions. Briefly, 100 μL of supernatant was removed and transferred into the corresponding wells of a clear 96-well plate in triplicates. Freshly prepared reaction mixture (100 μL) was added to each well and incubated at RT for 30 min, protected from light. The plate was read using a FlexStation3 microplate reader (Molecular Devices) at 490 nm. The average absorbance of each triplicate was determined and used to calculate cell viability percentage.

Glucose Consumption Assay. The glucose concentration in culture medium was measured using a glucose assay kit (Sigma, St. Louis, MO) following the manufacturer's instructions. Briefly, after cell treatment, 10 μL of cell culture medium was diluted to 100 μL with dH_2O. Assay reagent (200 μL) was then added to each sample. The color reaction was stopped with 200 μL of 12 N H_2SO_4. OD was measured at 540 nm. Glucose consumption was calculated using the starting glucose concentration in culture medium (1 mg/mL) minus the glucose concentration measured at the end of experiment. Glucose consumption was then normalized to protein level of the cells. Cell protein level was quantitated with Coomassie Plus (Thermo Scientific, Waltham, MA).

Western Blotting. Cell processing, SDS-PAGE, and membrane transfer have been described previously.[26] Briefly, HepG2 cells were either untreated or treated with reagents indicated for 2 h. Cells were lysed with RIPA buffer supplied with 1× Halt protease inhibitor cocktail with EDTA (Thermo Scientific, Waltham, MA) and 1× Halt phosphatase inhibitor cocktail (Thermo Scientific, Waltham, MA), separated by SDS-PAGE, and transferred to a polyvinylidene difluoride (PVDF) membrane, and endogenous proteins were detected by Western blot using specific antibodies.

RNA Extraction and Real-Time PCR. Total RNA was prepared from HepG2 cells using Trizol (Invitrogen, Grand Island, NY) and RNeazy kit following the manufacturer's instructions and as described previously.[27] The cDNA was generated from 2 μg of total RNA using High-Capacity cDNA Reverse Transcription kits (Applied Biosystems, Carlsbad, CA). The samples were then analyzed using TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA) in real-time quantitative PCR system Via7. The primers and probes included ACCB (Hs00153715), G6Pase (Hs00609178), LDLR (Hs00181192), PGC1α (Hs01016719), SIRT1 (Hs01009005), and 18S rRNA (Hs99999901) as a reference gene.

Glycogen Content. Glycogen was measured in HepG2 cells using a glycogen assay kit (BioVision, Milpitas, CA) following the manufacturer's instructions. Briefly, HepG2 cells growing in a 24-well plate were homogenized with 120 μL of dH_2O on ice. The homogenates were boiled for 5 min and spun at 13 000 rpm for 5 min to remove insoluble material. Supernatants (50 μL) were incubated with hydrolysis enzyme mix for 30 min at room temperature, and then 50 μL of the reaction mix was added to each sample for 30 min. OD was measured at 570 nm. For each sample, another set of aliquots (50 μL) without hydrolysis was also included as free glucose background. Glycogen content of the cells was calculated using total glucose level minus free glucose background of each sample and normalized to protein levels.

Glycolysis Assay with Seahorse XF96. Glycolysis was determined by measuring the extracellular acidification rate (ECAR) using a Seahorse XF96 analyzer (Seahorse Bioscience, North Billerica, MA), which assesses glycolysis and oxidation in living cells using extracellular flux methods.[28,29] HepG2 cells were seeded in XF 96-well plates at 12 × 10^4 cells/well for 24 h. Cells were starved in DMEM low-glucose overnight. On the day of the assay, cells were pretreated with or without RE or metformin for 2 h. At that time, the medium was changed to unbuffered serum-free DMEM (Seahorse Bioscience, North Billerica, MA) without glucose, and the plate was placed in a non-CO_2 incubator for 1 h. Using the analyzer, glycolysis was determined by measuring ECAR using a glycolysis stress kit (Seahorse Bioscience, North Billerica, MA). The measurement protocol used is as follows: after four baseline measurements, 10 mM glucose and 100 mM 2-deoxyglucose (2-DG), an inhibitor of glycolysis, were injected sequentially. For each injection, the ECAR value was monitored at four successive 4 min intervals, with 2 min intermeasurement mixing. The experiment was performed three times, and each condition had six replicates. ECAR was determined by plotting the acidification rate of the medium in the chamber as a function of time and normalized to baseline.

Statistical Analysis. Data were presented as mean ± standard deviation (SD). Data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test for multiple comparisons to the control group with GraphPad software (La Jolla, CA). The diminishing effects of PRARγ inhibitor GW9662 on rosemary glucose consumption (Figure 4) were tested for effects of "dose" and "subject" in a two-way ANOVA, followed by Tukey's multiple comparison analysis when required. Differences were considered significant when $p < 0.05$.

RESULTS

Characterization of RE. The RE prepared for this study was characterized by GC-MS, and the compounds observed are recorded in Table 1. The compounds represent three common classes of constituents routinely observed in rosemary: phenolic acids, diterpenes, and triterpenes. The main compounds present in RE include carnosic acid, ursolic acid, oleanolic acid, methyl carnosate, rosmarinic acid, betulinic acid, carnosol, rosmanol, and caffeic acid (Table 1). The component mixture and relative concentrations are consistent with data reported previously for *R. officinalis* samples and extracts.[30,31]

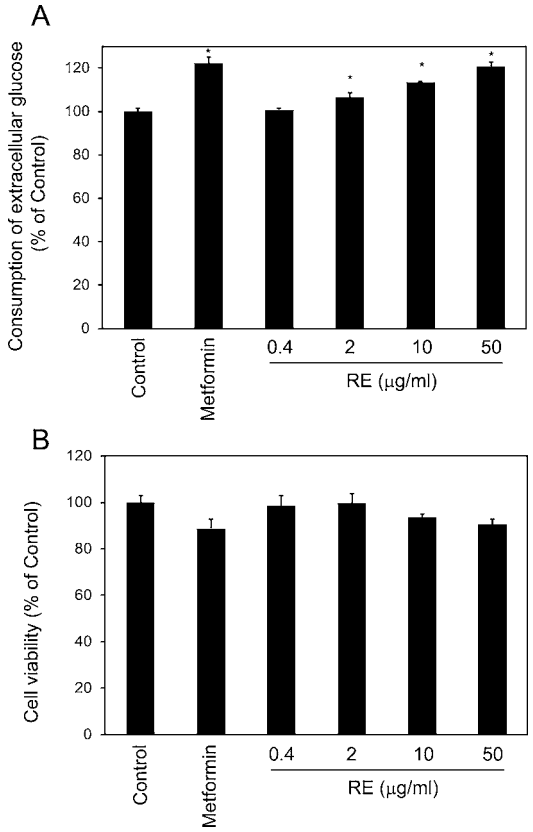

Figure 1. RE dose dependently increases glucose consumption in HepG2 cells. HepG2 cells were plated in 24-well plates overnight, then serum-starved for 24 h before treated with metformin (5 mM, positive control) or RE at concentrations indicated for 4 h. (A) Glucose consumption from culture medium after treatment as percentage of control. (B) Aliquots of culture medium were also collected, and the release of LDH was measured as an indicator of cell viability. Shown is a representative result, performed in duplicate with standard deviations, from three independent experiments. * indicates statistical significance ($p < 0.05$).

RE Increases Glucose Consumption in HepG2 Cells. To understand the potential roles of RE in glucose metabolism, we first examined its ability to regulate glucose consumption in HepG2 cells. Metformin, an antidiabetic drug of the biguanide class, was used as a positive control. As shown in Figure 1A, RE at 2, 10, and 50 μg/mL dose dependently and significantly increased glucose consumption of HepG2 cells 6%, 13%, and 21%, respectively, compared to the control group. RE at 0.4 μg/mL did not show any effect (Figure 1A). Metformin at 5 mM concentration also dramatically increased glucose consumption by 22% (Figure 1A), which is consistent with previous publications.[20,32] The effects on glucose consumption were not related to cytotoxicity of RE, as the LDH assay showed a survival rate of ≥90% for all the concentrations tested (Figure 1B). The data confirmed that RE increased glucose consumption in HepG2 cells from extracellular medium.

RE Activates AMPK Pathway in HepG2 Cells. To understand whether RE regulates liver metabolism via AMPK pathway, we examined the phosphorylation of AMPK by tumor suppressor LKB1 at Thr-172, which is required for AMPK activation.[33−35] As demonstrated in Figure 2, treatment with RE (10 and 50 μg/mL) significantly increased phosphorylation of Thr-172 of AMPK compared to controls. Metformin also dramatically increased the phosphorylation of AMPK (Thr-172) (Figure 2). The increase in phosphorylation was not attributed to the protein expression levels, as total AMPK levels in these groups were constant (Figure 2).

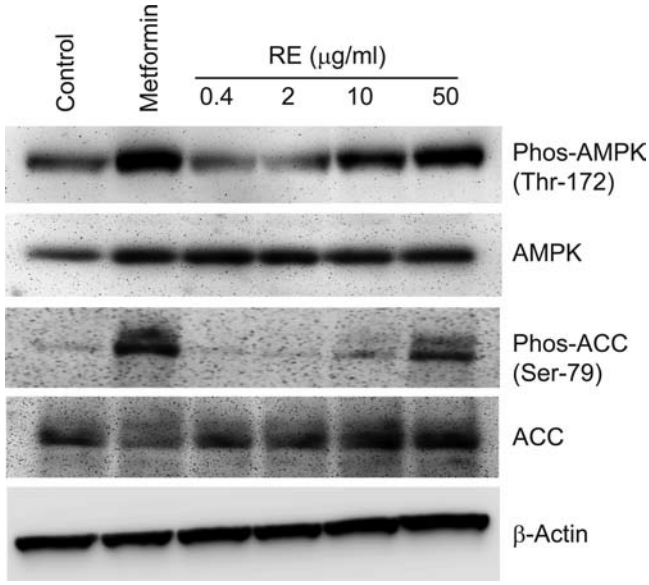

Figure 2. RE activates AMPK pathway. HepG2 cells were plated in six-well plates overnight, then serum-starved for 24 h before treated with metformin (5 mM) or RE at concentrations indicated for 2 h. The cells were lysed, and the lysates were analyzed by Western blotting using antibodies against phospho-AMPKα (Thr-172), AMPKα, phospho-ACC (Ser-79), ACC, and β-actin.

One way AMPK influences fatty acid metabolism is by phosphorylating and inhibiting acetyl-CoA carboxylase (ACC) at Ser-79.[36−38] ACC catalyzes the carboxylation of acetyl CoA to malonyl CoA, which is critical for fatty acid synthesis and β-oxidation. We examined the phosphorylation of ACC at Ser-79. RE, as well as metformin, markedly increased the phosphorylation of endogenous ACC while only affecting the total ACC marginally, if at all (Figure 2). The level of β-actin, an internal control, was not affected by RE or metformin treatment (Figure 2). Therefore, our data indicated that components of RE are capable of activating the AMPK−ACC pathway.

RE Modulates the Expression of Genes Involving Metabolism in HepG2 Cells. To address whether RE can influence lipid and glucose metabolism through transcriptional regulation, we investigated the expression of a series of genes involved in metabolism, including glucose-6-phosphatase (G6Pase), acetyl-CoA carboxylase β (ACCB), low-density lipoprotein receptor (LDLR), SIRT1, and PPARγ coactivator-1α (PGC1α). RE at 10 and 50 μg/mL decreased the mRNA level of G6Pase 32% and 65%, respectively, compared to control (Figure 3). Metformin treatment lowered the expression of G6Pase 42%, consistent with an earlier report that metformin-induced AMPK activation down-regulated G6Pase in primary hepatocytes.[21] The expression of ACCB was reduced 32% after treatment with 50 μg/mL of RE (Figure 3). Taken together with our earlier findings on ACC phosphorylation, it appears that RE negatively regulates ACC activity on both transcription and post-translation levels. LDLR mediates the endocytosis of cholesterol-rich LDL. As shown in Figure 3, the mRNA level of LDLR was markedly increased (2-fold) by 50 μg/mL RE treatment, indicating a potential role of rosemary in lipoprotein signaling. Metformin also increased LDLR expression (Figure 3). Furthermore, similar to

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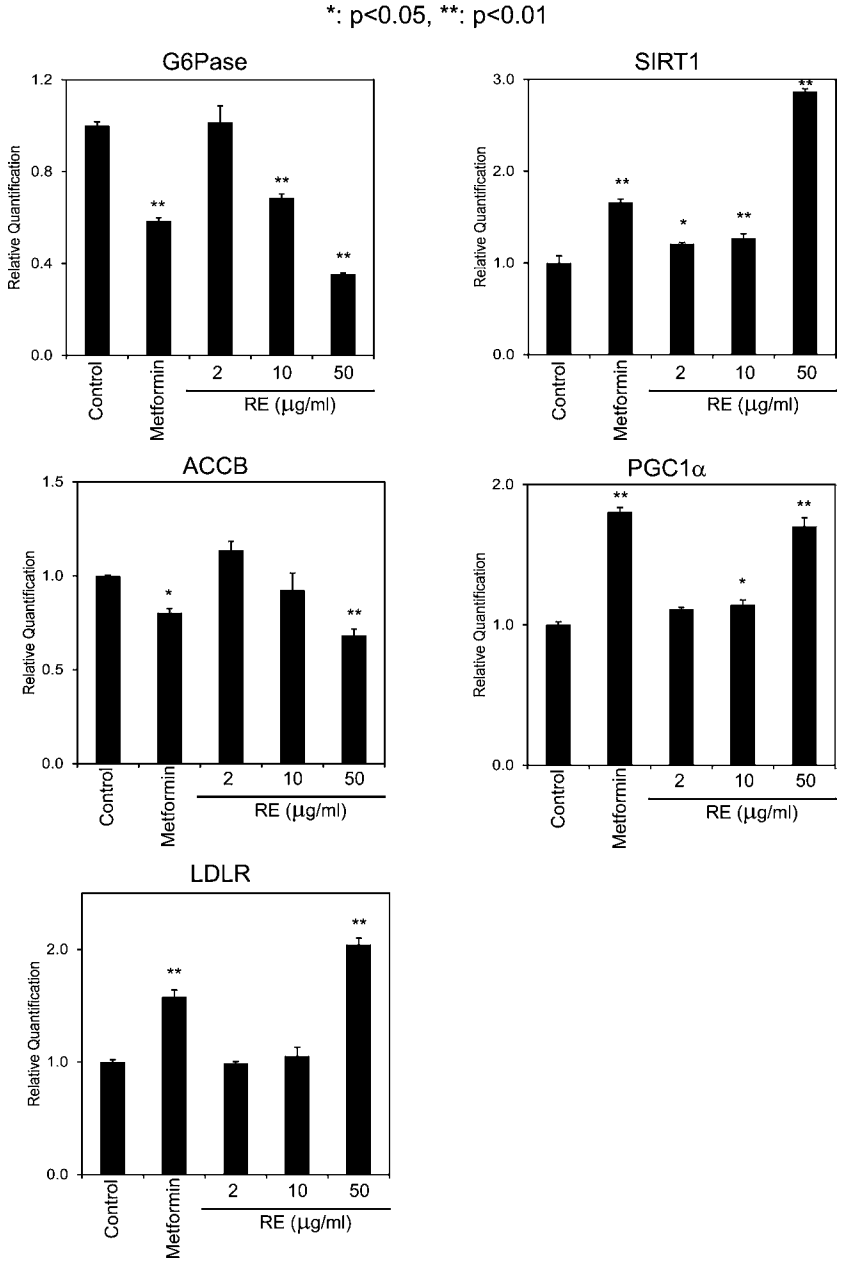

Figure 3. RE modulates expression of metabolism genes. HepG2 cells were plated in six-well plates overnight, then serum-starved for 24 h before treated with metformin (5 mM) or RE at concentrations indicated for 4 h. Total RNA was extracted from the cells, and the expression of G6Pase, ACCB, LDLR, SIRT1, and PGC1α was analyzed by real-time PCR and normalized to 18S RNA. Representative results from three independent experiments are shown. * indicates statistical significance ($*p < 0.05$; $**p < 0.01$).

metformin, RE (at 50 μg/mL) increased the mRNA levels of both SIRT1 (2.9-fold) and PGC1α (1.7-fold) in HepG2 cells (Figure 3). Overall, these data suggested that RE may regulate glucose and lipid metabolism through transcription of multiple pathways.

PPARγ Inhibitor GW9662 Diminishes the Effects of RE on Glucose Consumption. Our data suggested that RE increased the transcription of PGC1α (Figure 3). Therefore, PPARγ may be a potential target of rosemary to influence metabolism in liver cells. To test this hypothesis, specific PPARγ antagonist GW9662 was incubated with HepG2 cells overnight at 0, 2, and 10 μM. Then consumption of extracellular glucose by HepG2 cells treated with different concentrations of RE or metformin was measured. As expected, incubation of metformin or RE (50 μg/mL) increased glucose consumption significantly (Figure 4, open bars). Preincubation of GW9662 dose-dependently reduced glucose consumption compared to metformin or RE alone groups (Figure 4, comparing open, gray, and black bars of metformin group or RE groups). GW9662 did not affect the basal level of glucose consumption in the control group significantly (Figure 4). Altogether, our data, as well as a previous publication,[10] suggested that PPARγ is involved in the regulation of liver energy metabolism by rosemary.

RE Reduces Glycogen Content and Increases Glycolysis in HepG2 Cells. Our data suggested that RE increased glucose consumption from extracellular medium (Figure 1). In an effort to understand the destination of the increased glucose in cells, we examined the glycogen content and glycolysis activity after rosemary extract treatment. As shown in Figure 5,

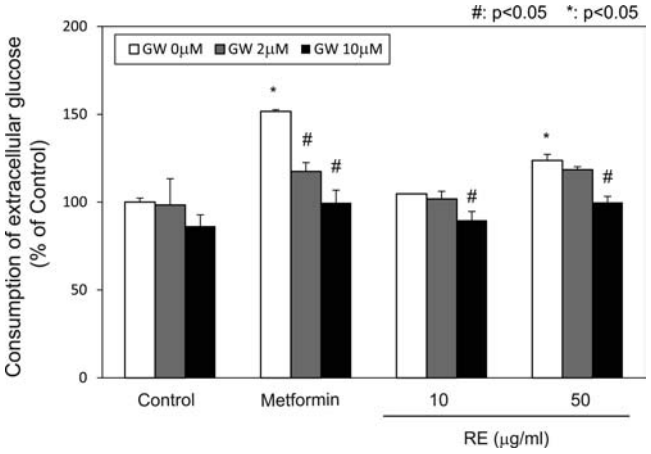

Figure 4. PPARγ inhibitor GW9662 diminished rosemary effects on glucose consumption. HepG2 cells were plated in 24-well plates overnight. The next day, serum-free medium with or without GW9662 at 2 or 10 μM was added to the cells for 16 h. Then the cells were treated with metformin (5 mM) or RE at concentrations indicated for 4 h. Glucose concentrations in culture medium were measured. Shown is a representative result, performed in duplicate with standard deviations, from three independent experiments. * indicates statistically significant difference among samples without GW9662 treatment (open bars, $p < 0.05$); # indicates statistically significant difference among samples treated with either metformin or RE plus different concentrations of GW9662 (comparing open, gray, and black bars grouped together, $p < 0.05$).

Table 1. Major Phytochemicals Identified in Albanian Rosemary (*Rosmarinus officinalis L.*)

compound	concentration (mg/100g dry ground spice)	concentration (μg/mg RE)
betulinic acid	960	19.2
caffeic acid	125	2.5
carnosic acid	2420	48.4
carnosol	340	6.8
methyl carnosate[a]	1170	23.4
oleanolic acid	1390	27.8
rosmanol[b]	200	4
rosmarinic acid	960	19.2
ursolic acid	2220	44.4

[a]No standard available. Amount calculated from a ratio of GC peak area for methyl carnosate to the area of carnosic acid peak. [b]No standard available. Amount calculated from a ratio of GC peak area for rosmanol to the area of carnosol peak.

treatment of RE reduced glycogen content inside the cells in a dose-dependent manner. RE (50 μg/mL) decreased glycogen content by 67% compared to controls. A similar effect was seen with metformin (Figure 5).

Glycolysis was determined by measuring ECAR. HepG2 cells were serum-starved overnight and then treated with metformin or different concentrations of RE for 2 h. The kinetics of ECAR reading and area under the curve calculation are shown in Figure 6A and 6B, respectively. Injection of glucose dramatically increased ECAR value (Figure 6B). HepG2 cells exhibit a dose-dependent increase in ECAR value in response to RE treatment, which is statistically significant ($p < 0.05$), compared to glucose injection alone (Figure 6B). As expected, metformin also markedly increased ECAR (Figure 6B). Injection of 100 mM 2-DG abolished ECAR response induced by glucose injection (Figure 6A). Therefore, rosemary likely induces glucose consumption by increasing glycolysis in HepG2 cells.

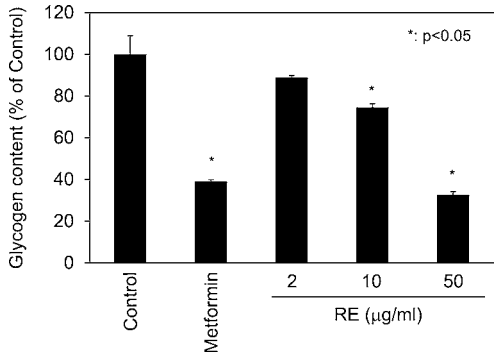

Figure 5. RE decreases glycogen content in HepG2 cells. HepG2 cells were plated in 24-well plates overnight, then serum-starved for 24 h before treatment with metformin (5 mM) or RE at concentrations indicated for 4 h. The cells were lysed, and glycogen levels in the lysates were measured. Free-glucose background in the lysates was subtracted (see Materials and Methods section). Representative results from three independent experiments are shown. * indicates statistical significance ($*p < 0.05$).

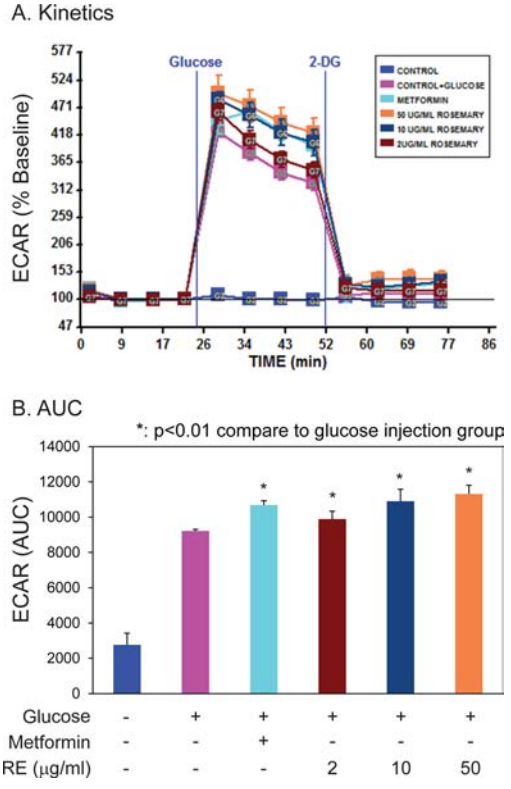

Figure 6. RE increases glycolysis in HepG2 cells. HepG2 cells were plated in 96-well Seahorse cell culture plates overnight, then serum-starved for 24 h before treated with metformin (5 mM) or RE at concentrations indicated for 2 h. ECAR was then measured using an XF-96 machine following manufacturer instructions. (A) Kinetics measurement. (B) Area under the curve measurement. Representative results from three independent experiments are shown. * indicates statistical significance ($*p < 0.05$).

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DISCUSSION

In vivo studies found that rosemary extracts can lower fasting plasma glucose and cholesterol levels, as well as mitigate weight gain in mice fed a high fat diet.[14,15,17] Our *in vitro* data in HepG2 cells demonstrated that RE activates the AMPK and PPAR signaling pathways, up-regulates LDLR, SIRT1, and PGC1α, and down-regulates ACCB and G6Pase. In addition, RE increases liver cell glucose consumption and glycolysis. Overall, these findings suggested that RE favorably regulates lipid and glucose metabolism through activation of multiple pathways.

The present studies explored the potential mechanisms of RE in HepG2 hepatocytes in comparison to metformin. Metformin, which treats hyperglycemia and hyperlipidemia, among others, is one of the first-line therapeutic agents for type-2 diabetes patients.[32] It has been reported that metformin increases insulin sensitivity, enhances peripheral glucose uptake, and increases fatty acid oxidation.[39,40] Our results showed there are some overlaps between rosemary and metformin in terms of pathways activated in liver cells, namely, AMPK, SIRT1, and PPARs. AMPK is an important target of metformin action.[21,34] Our data illustrated that RE by itself increased phosphorylation of AMPK and its substrate ACC in a dose-dependent manner (Figure 2). Phosphorylation of Ser-79 of ACC inhibits its catalytic activity. Furthermore, treatment with RE decreased the mRNA level of ACCβ in liver cells (Figure 3). These combined effects are expected to result in suppression of the conversion of acetyl CoA to malonyl CoA by ACC. The function of malonyl CoA is 2-fold: an essential substrate of fatty acid biosynthesis and an inhibitor of carnitine palmitoyltransferase 1 (CPT1), which is critical for entry of long-chain fatty acids into mitochondria and their subsequent oxidation.[41] Therefore, RE may potentially help not only block the rate-limiting step in fatty acid synthesis but also augment β-oxidation by inhibiting ACC.

RE induced the expression of SIRT1 and PGC1α (Figure 3). SIRT1, an NAD^+-dependent protein deacetylase, is also widely regarded as a fuel-sensing molecule.[42] Induction of SIRT1 expression increases insulin sensitivity.[43] The mechanism in regulation of SIRT1 on metabolic responses is due to its ability to deacetylate proteins, such as PPARs and PGC1α.[32,44,45] A recent publication demonstrated that hepatocyte-specific deletion of SIRT1 in mice severely decreased fatty acid β-oxidation, whereas overexpression of SIRT1 had the opposite effects.[46] Therefore, the increased expression of SIRT1 by rosemary may also increase liver fatty acid oxidation. As a transcription coactivator, PGC1α plays a critical role in the maintenance of mitochondrial function, thermogenesis, and energy homeostasis, mainly by activating a variety of nuclear receptors, including PPARα, PPARγ, PPARδ, and liver X receptors (LXR).[47] Rosemary-induced PGC1α increase potentially leads to activation of PPAR pathways. In fact, our data showing that the specific PPARγ antagonist GW9662 inhibits rosemary's effects on glucose consumption (Figure 4) confirms that PPAR pathways are involved in rosemary signaling, probably through regulating PGC1α. RE also increased the expression of liver LDLR by 2-fold (Figure 3). Considering the fact that liver accounts for removal of ~70% of LDL from circulation, this finding may provide a potential mechanism for some *in vivo* observations that RE can lower blood cholesterol level.[13] Taken together, activation of the AMPK−ACC pathway and up-regulation of SIRT1/PGC1α strongly suggested a role for rosemary in regulating fatty acid synthesis and β-oxidation.

In terms of glucose metabolism, we looked into the effects of rosemary on gluconeogenesis, glycogen level, and glycolysis. RE transcriptionally regulates the expression of G6Pase (Figure 3). G6Pase catalyzes the final step in gluconeogenesis and therefore plays a key role in the homeostatic regulation of blood glucose levels. Both RE and metformin significantly decreased the expression of G6Pase in our experiments, suggesting their roles in suppressing gluconeogenesis. Similar observations have been reported with metformin previously.[20,48] Our study demonstrated for the first time that RE induced glycolysis in HepG2 cells (Figure 6). This effect is probably also the results of AMPK activation. Previous publications have shown that AMPK activation is capable of inducing glycolysis[49−51] and down-regulating glycogen synthesis.[49] We also observed a decrease in glycogen content after RE and metformin treatment (Figure 5). This is likely due to reduced glycogenesis, as AMPK is capable of phosphorylating glycogen synthase (GS) and inhibiting GS activity.[22] Overall, these investigational studies demonstrate the roles of rosemary in glucose metabolism as increasing glycolysis, while decreasing gluconeogenesis and glycogen content.

In summary, treatment with RE could activate energy sensing molecules, including AMPK and SIRT1, which in turn induce catabolic pathways such as fatty acid oxidation and glycolysis and inhibit anabolic pathways such as fatty acid and glycogen synthesis and gluconeogenesis. Collectively, our current studies and previous publications confirmed the roles of rosemary in lipid and carbohydrate metabolisms and pointed out that rosemary may serve as a potential hypoglycemic and hypolipidemic agent.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED:

ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; CPT1, carnitine palmitoyltransferase 1; G6Pase, glucose-6-phosphatase; LDH, lactate dehydrogenase; LDLR, low-density lipoprotein receptor; PGC1α, peroxisome proliferator-activated receptor γ coactivator 1α; SIRT1, sirtuin 1

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