Resveratrol inhibits the mevalonate pathway and potentiates the antiproliferative effects of simvastatin in rat theca-interstitial cells

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Objective: To examine the mechanisms of action of resveratrol and its interaction with simvastatin on growth and the mevalonate pathway in rat theca-interstitial cells.
Design: In vitro study.
Setting: Research laboratory.
Animal(s): Immature Sprague-Dawley female rats.
Intervention(s): Theca-interstitial cells were cultured in the absence or presence of resveratrol, simvastatin, mevalonic acid, farnesyl pyrophosphate, and/or geranylgeranyl pyrophosphate.
Main Outcome Measure(s): DNA synthesis was assessed by thymidine incorporation assay; 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) expression and activity were evaluated with the use of quantitative real-time polymerase chain reaction, Western blot analysis, and HMGCR activity assay. Cholesterol synthesis was determined by the conversion of [^{14}C]-acetate to [^{14}C]-cholesterol.
Result(s): Resveratrol potentiated the simvastatin-induced inhibition on cell proliferation in a concentration-dependent manner. Inhibitory effects of resveratrol were partly abrogated by the addition of mevalonic acid, farnesyl pyrophosphate, and geranylgeranyl pyrophosphate. Resveratrol reduced HMGCR expression and activity, and decreased cholesterol synthesis. In contrast, simvastatin inhibited HMGCR activity with a compensatory increase in HMGCR expression. Resveratrol counteracted this effect of simvastatin on HMGCR expression but augmented the simvastatin-induced inhibition on HMGCR activity and cholesterol synthesis.
Conclusion(s): Resveratrol inhibits the mevalonate pathway via distinctly different mechanisms than statins. These observations demonstrate a novel mechanism of action of resveratrol and underscore the potential translational/clinical relevance of resveratrol interactions with simvastatin. (Fertil Steril® 2011;96:1252–8. ©2011 by American Society for Reproductive Medicine.)

Key Words: Cholesterol, HMG-CoA reductase, mevalonic acid, farnesyl pyrophosphate, geranylgeranyl pyrophosphate, ovarian theca-interstitial cells, resveratrol, simvastatin

The mevalonate pathway provides cells with essential products such as cholesterol and substrates for isoprenylation that are important for cell growth and differentiation (1). Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (HMGCR), the rate-limiting enzyme of the mevalonate pathway (1). The inhibition of HMGCR by statins leads to reduced production of cholesterol and isoprenoids: farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) (2). FPP and GGPP are required for the isoprenylation of proteins such as small guanine triphosphatases that are important in signal transduction pathways regulating proliferation.

Polycystic ovary syndrome (PCOS) is a common endocrinopathy affecting 6.5%–6.8% of women of reproductive age (3, 4). Ovaries of women with PCOS are typically enlarged, with prominent hyperplasia of theca-interstitial cells producing excessive amounts of androgens (5, 6). PCOS is also associated with increased oxidative stress and systemic inflammation, as evidenced by elevated levels of C-reactive protein (7), advanced glycation end products (8), and reactive oxygen species (9). In clinical trials, statins improved the lipid profile, reduced androgen levels, and decreased systemic inflammation in women with PCOS (10, 11). In our previous studies, statins reduced theca-interstitial cell growth (12) and androgen production (13).

Resveratrol (*trans*-3,5,4′-trihydroxystilbene) is a natural polyphenol found in grapes, nuts, and red wine and has anticarcinogenic, anti-inflammatory, antioxidant, and cardioprotective properties (14). Our recent study has demonstrated that resveratrol inhibits ovarian theca-interstitial cell proliferation and induces apoptosis (15). Resveratrol exerts cardioprotective effects by suppressing low-density lipoprotein oxidation (16) and decreasing cholesterol levels (17). Furthermore, resveratrol reduces tumor necrosis factor–α, C-reactive protein, and reactive oxygen species levels (18); protects against advanced glycation end product–induced cholesterol accumulation in macrophages (19); lowers insulin-like growth factor I (20) and glucose levels; and improves insulin sensitivity (21). These properties of resveratrol are relevant to PCOS, which is associated with increased

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cardiovascular risk factors, including dyslipidemia, insulin resistance, systemic inflammation, and oxidative stress (7, 22).

In vivo, resveratrol improved the lipid profile and reduced hepatic HMGCR mRNA expression and activity in hamsters (17) and mice (23). However, in contrast, an in vitro study failed to detect any significant effect of resveratrol on cholesterol synthesis and HMGCR activity in normal rat hepatocytes (24).

This study evaluated the effects of resveratrol and its interaction with simvastatin on proliferation and the mevalonate pathway in rat ovarian theca-interstitial cells. Because the actions of resveratrol may be related to a decreased availability of downstream products of the mevalonate pathway, including mevalonic acid (MA), FPP, and GGPP, the study also examined whether these molecules modulate the resveratrol-induced suppression of cell proliferation.

MATERIALS AND METHODS

Cell Culture

Theca-interstitial cells were isolated from immature rat ovaries as previously described (25). The cells were counted, and the viability assessed by the trypan blue exclusion test was 90%–95%. The cells were cultured at 37°C in a humidified atmosphere of 95% air/5% CO_2, in serum-free McCoy's 5A medium (supplemented with antibiotics, 0.1% BSA, and 2 mM L-glutamine). The cells were incubated without (control) or with resveratrol, simvastatin, MA, FPP, and/or GGPP for 24–48 hours. Simvastatin was hydrolyzed to break the lactone ring. All chemicals were purchased from Sigma Chemical Co. All treatments and procedures were carried out in accordance with accepted standards of humane animal care as outlined in the NIH *Guide for the Care and Use of Laboratory Animals* with a protocol approved by the Institutional Animal Care and Use Committee at the University of California, Davis.

Proliferation Assay

Cells were incubated for 48 hours in 96-well culture plates. DNA synthesis was determined by a thymidine incorporation assay. [^{3}H] thymidine (1 μCi/well) was added during the last 24 hour of culture. The cells were then harvested using a multiwell cell harvester (PHD Harvester; Cambridge Technology, Inc.). Radioactivity was measured using a liquid scintillation counter, Wallac 1409 (PerkinElmer).

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction

Cells were treated without (control) or with resveratrol (30–100 μM) and/or simvastatin (10 μM) for 24–48 hours. RNA was isolated using the MagMAX-96 Total RNA Isolation Kit (Applied Biosystems). Reverse transcription of total RNA to cDNA was performed using SuperScript III First-Strand Synthesis system for real-time polymerase chain reaction (PCR) (Invitrogen). SYBR Green detection was employed and quantitative real-time PCR reactions were performed in triplicate using SYBR Green PCR Master Mix (Applied Biosystems).

Several potential endogenous controls were evaluated, including hypoxanthine phosphoribosyltransferase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β_2-microglobulin (B2M) and β-actin, to identify the gene whose expression remains the most stable under our experimental conditions. This was accomplished using the NormFinder algorithm (26) (http://www.mdl.dk/publicationsnormfinder.htm), which identified hypoxanthine phosphoribosyltransferase mRNA as the most stable and hence used it as the endogenous reference. The relative amount of HMGCR mRNA was normalized to hypoxanthine phosphoribosyltransferase. The primer sequences were as follows: rat HMGCR forward (5′-TGT GGG AAC GGT GAC ACT TA-3′) and reverse (5′-CTT CAA ATT TTG GGC ACT CA-3′), and rat hypoxanthine phosphoribosyltransferase forward (5′-TTG TTG GAT ATG CCC TTG ACT-3′) and reverse (5′-CCG CTG TCT TTT AGG CTT TG-3′).

Western Blot Analysis

Cells were incubated without (control) or with resveratrol (50 μM) and/or simvastatin (10 μM) for 48 hours. At the end of treatments, cell lysates were prepared as previously described (27). Protein concentrations were determined using Bio-Rad Protein Assay (Bio-Rad). For immunoblot analysis, 100 μg of protein was resolved in 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to a nitrocellulose membrane by electroblotting. Blots were blocked using blocking buffer (LI-COR Biosciences), incubated with primary antibody to HMGCR (1:200; Millipore) and β-actin (1:1000; Sigma Aldrich), washed and incubated with the secondary antibodies (1:5000; LI-COR Biosciences). Blots were developed with the Odyssey Infrared Imaging System (LI-COR Biosciences). Band intensities were normalized to β-actin.

HMGCR Activity Assay

The microsomes were isolated and HMGCR activity was determined by measuring the conversion of [3-^{14}C]-HMG-CoA to [^{14}C]-mevalonic acid lactone (28). Mevalonic acid lactone bands were visualized by iodine vapor staining, the bands were scraped, and the radioactivity was determined using a scintillation counter with ^{14}C channel detector.

Cholesterol Biosynthesis Assay

Cholesterol biosynthesis was determined by the conversion of [^{14}C]-acetate to [^{14}C]-cholesterol (29, 30). Briefly, cells were treated with sodium [2-^{14}C]-acetate (50–60 μCi/mmol), resveratrol, and/or simvastatin for 24 hours. The extracts were separated by thin-layer chromatography in benzene/ethyl acetate (9:1). Samples containing cold cholesterol and [^{3}H]-cholesterol were applied to the thin-layer chromatography plate to aid in the identification of cholesterol bands. Cholesterol bands were visualized with the use of primuline under the ultraviolet light, cut from the thin-layer chromatography plate, and transferred into scintillation vials for radioactivity measurements. The amount of [^{14}C]-cholesterol detected was corrected for recovery with [^{3}H]-cholesterol.

Statistical Analysis

Statistical analysis was performed with JMP 8.0 software (SAS). Comparisons between the groups were determined by analysis of variance followed by post-hoc Tukey honestly significant difference test. Normality of distribution was assessed by the Shapiro-Wilk W test. In the absence of normality, nonparametric testing (Kruskal-Wallis) was used. When appropriate, data were logarithmically transformed. Baseline data are expressed as means ($\pm$SEM). A value of $P<.05$ was considered statistically significant.

RESULTS

Resveratrol and Simvastatin Inhibit Proliferation in a Concentration-Dependent and Additive Fashion

To test the hypothesis that resveratrol may potentiate the inhibitory effects of simvastatin on cell proliferation, cells were cultured with resveratrol (30 and 50 μM) and/or simvastatin (1 and 10 μM). The uptake of thymidine, as an index of DNA synthesis, was measured. As shown in Figure 1A, resveratrol and simvastatin individually inhibited DNA synthesis in a concentration-dependent manner ($P<.001$). Resveratrol (30 μM) potentiated the inhibitory effect of simvastatin (1 and 10 μM) on cell proliferation, by 36% ($P<.001$) and 8% ($P<.001$), respectively, when compared to the inhibition observed in the presence of simvastatin alone. In a similar fashion, resveratrol (50 μM) potentiated the simvastatin-induced inhibition on cell proliferation, by 60% ($P<.001$) and 14.9% ($P<.001$), respectively, when compared to the inhibition observed in the presence of simvastatin (1 or 10 μM) alone.

Effects of Resveratrol on Proliferation are Partly Reversed by Products of the Mevalonate Pathway

To study whether the resveratrol-induced reduction in DNA synthesis is related to a depletion of downstream products of the mevalonate pathway, cells were cultured without (control) or with resveratrol (30 and 50 μM) and/or MA (100 μM), FPP (30 μM), and GGPP (30 μM) for 24 hours. As shown in Figure 1B, in the

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