SUPPLEMENTAL DATA

Genistein, Resveratrol and AICAR Induce Cytochrome P450 4F2 Expression through an AMPK-dependent Pathway

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Running Title: AMPK regulation of CYP4F2

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TABLE S1 PCR primer sets used in this study.

Gene	Oligonucleotide(5' to 3')	Accession #	Location(bp)
CYP4F2	CACCATGAGATCCTCCTGCATATT	NM_001082.3	756-779
	TCTCTTTAGGCTCACGGTCCTTCA		1135-1158
CYP4F3	CACCAGCAGATCCTCCTGTACATA	NM_000896	756-779
	TCTCTTTAGGCTCACGGTCCTTCA		1158-1135
CYP4F11	ATACCACCTTGCAAAGCACC	NM_021187	1481-1500
	TGGGTGGGTAGGACAGTCACT		2029-2049
CYP4F12	GCTGCCATTGCACCCAAGGATAAT	NM_023944	404-427
	ACAGAAAGTCCATGTGCTGGAGGA		777-800
hAMPKα1	TTGCGTGTACGAAGGAAGAA	NM_006251.5	1333-1352
	CCTTGAGCCTCAGCATCTGAA		1547-1527
hAMPKα2	GGCAATTACGTGAAAATGAGC	NM_006252	1413-1433
	GGTGAAACTGAAGACAATGTGCTT		1660-1630
hPPIA[a]	CCATCTATGGGGAGAAATTTGA	NM_021130	274-295
	GAGTTGTCCACAGTCAGCAATG		536-515
hHMGCR	GGCTGGGAGCATAGGAGGCTAC	NM_000859	2276-2297
	TCTTGGTGCAAGCTCCTTGGAG		2685-2706
hLDLR	CCTGCAAGGCTGTGGGCTCC	NM_000527	1340-1359
	GCCCTTGGCTTGGAGCCGTT		1711-1730
hSHP	GAAGATTCTGCTGGAGGAGCCC	NM_021969	386-407
	GTCACCTGAGCAAAAGCATGTCC		782-804

[a] HMGCR, HMG-CoA reductase; LDLR, low density lipoprotein receptor; PPIA, cyclophilin A; SHP, small heterodimer partner

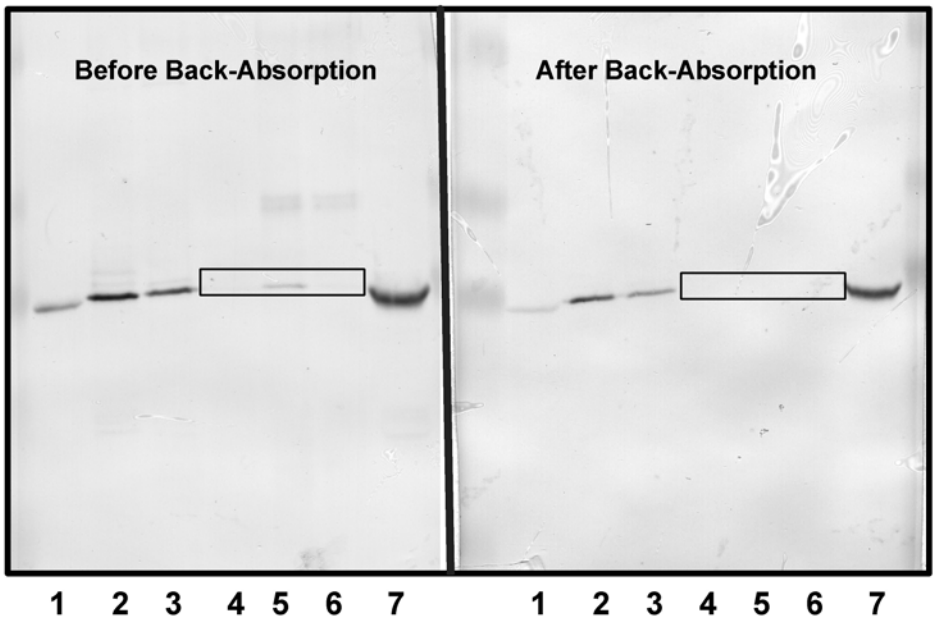

Figure S1. Immunoreactivity of P450 4F2/3 antibody.

Human liver microsomes and recombinant human CYP4F2, CYP4F11 and CYP4F12 *Sf*9 lysates were prepared as described in "Methods" and subjected to immunoblotting with anti-human P450 4F2/3 IgG. Blot lane identification: 1= human liver microsomes, UP878, 15.0 μg; 2 = 4F2 *Sf*9 lysates, 0.5 μg; 3 = 4F3B *Sf*9 lysates, 0.5 μg; 4 = 4F11 *Sf*9 lysates, 0.5 μg; 5 = 4F12 *Sf*9 lysates, 1.0 μg; 6 = mixture of 4F12 and 4F11 *Sf*9 lysates, 1.0 μg; and 7 = human liver microsomes, UC8911, 15.0 μg. The immunoblot was developed using immunochemical staining. The left blot shows that anti-P450 4F2/3 IgG does not exhibit extensive cross-reactivity with either P450 4F11 and/or P450 4F12.

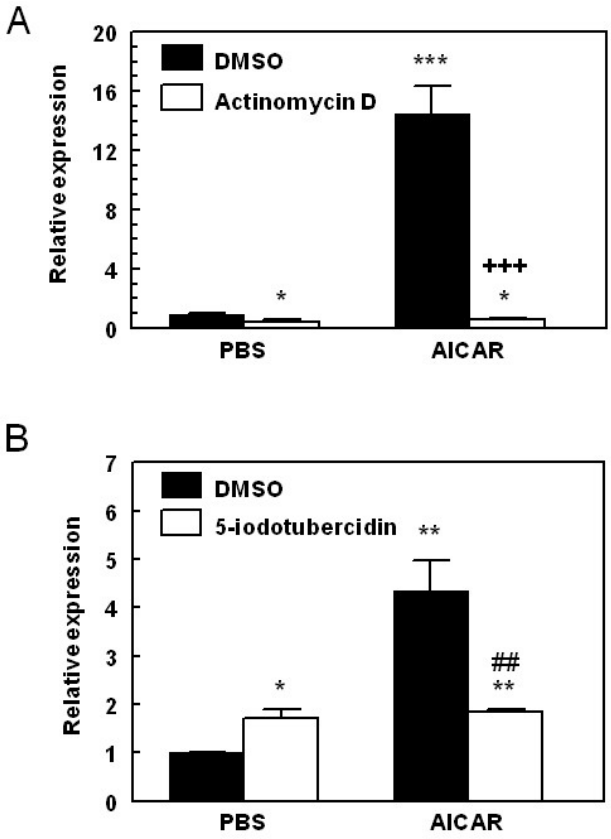

Figure S2. Actinomycin D (A) and 5-iodotubercidin (B) inhibit the AICAR stimulated PPIA normalized *CYP4F2* mRNA expression in HepG2 cells.

(Panel A) Actinomycin D, an inhibitor of gene transcription, was added with 0.5 mM AICAR at a final concentration of 5 μg/ml. (Panel B) 5-iodotubercidin, a thymidine kinase inhibitor which inhibits the conversion of AICAR to ZMP, was administered with 0.5 mM AICAR to HepG2 cells at 10 μM final concentration. Total RNA was isolated for qPCR to assess CYP4F2 and PPIA mRNA expression levels after a 24 h drug treatment. The *CYP4F2* mRNA levels were normalized to PPIA mRNA levels. For each independent experiment, triplicate samples were used for each treatment and triplicate determinations were performed for each sample. For each individual experiment, the fold change was determined by comparison to the mean value obtained from cells treated with vehicle controls which was set as 1. Means and standard errors were determined from three independent experiments. Statistically significant differences compared to vehicle controls are depicted by the asterisks, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$; +++: significant difference between PBS and actinomycin D in the presence of AICAR, $p<0.001$, ##: significant difference between DMSO and 5-iodotubercidin treatments in the presence of AICAR, $p<0.01$.

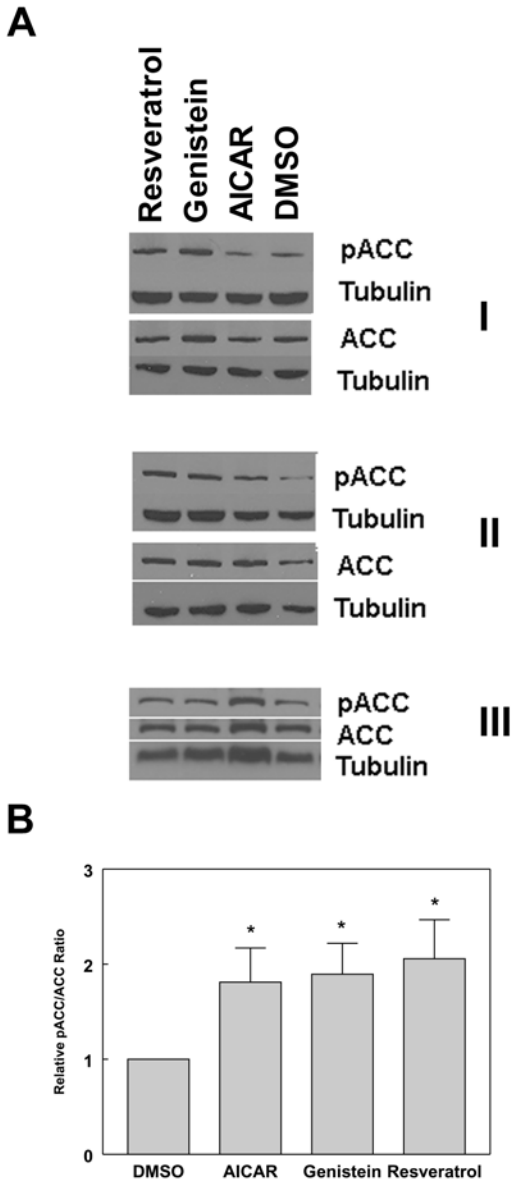

Figure S3. AMPK activators increase the phosphorylation of Acetyl-CoA carboxylase.

Whole cell lysates (50 µg) from HepG2 cells treated with genistein (100 µM), resveratrol (75 µM), AICAR (0.5 mM) or DMSO for 2 h were prepared as described (Nystrom and Lang, 2008). PhosphoSTOP™ phosphatase inhibitor cocktail and Complete™ protease inhibitor cocktail (Roche Applied Science) were also included during the preparation of lysates. Immunoblotting was performed using 10% NuPAGE Bis-Tris gels (Invitrogen). Nitrocellulose membranes were incubated with ..anti-phospho-Ser^{79} acetyl-CoA carboxylase 1 (pACC), anti-total ACC (ACC) and anti-β-tubulin (Tubulin) antibodies overnight at 4°C according to the manufacturer's instructions. After reaction with peroxidase-conjugated anti-rabbit IgG, the blots were developed using chemiluminescence (Western Lightening Plus, Perkin Elmer, Boston, MA). Three independent biological experiments were performed, and the x-ray films of the immunoblots are shown in panel A. X-ray films of the immunoblots were scanned, and the signals were analyzed with ImageQuant 5.2 software (GE Healthcare, Piscataway, NJ), and the results are shown in panel B. For experiment I and II, pACC and total ACC immunoblot analysis was performed on separate membranes. pACC and ACC signals were first normalized against that of tubulin. The ratio of pACC/ACC was then determined by comparing the tubulin normalized pACC over the tubulin normalized ACC. For experiment III, the pACC and total ACC immunoanalysis was performed on the same membrane. The ratio of pACC/ACC was determined by comparing the immunoreactive intensity of pACC over that of ACC. The ratio of pACC/ACC from the HepG2 cells treated with test compounds were compared to the corresponding DMSO controls, and are expressed as relative pACC/ACC ratio. The means and standard errors were determined from these three independent experiments shown in panel A. Statistically significant differences from DMSO controls are indicated as: *, $p<0.05$.

Reference

Nystrom GJ and Lang CH (2008) Sepsis and AMPK Activation by AICAR Differentially Regulate FoxO-1, -3 and -4 mRNA in Striated Muscle. *Int J Clin Exp Med* **1**:50-63.

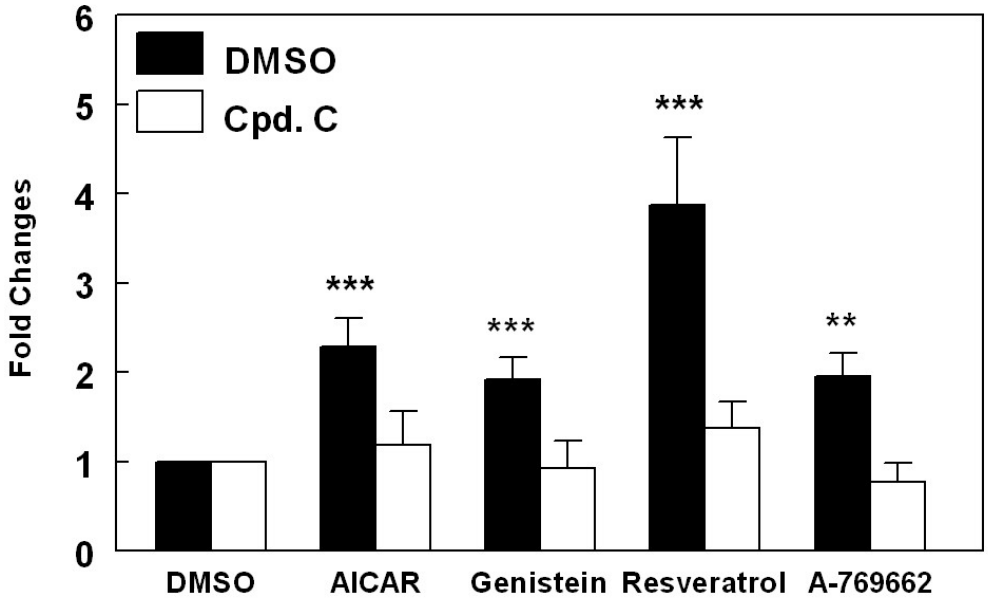

Figure S4. Compound C, an AMPK inhibitor, blocks stimulation of PPIA normalized small heterodimer partner (SHP) mRNA expression by genistein, resveratrol, AICAR and A-769662.

HepG2 cells were pretreated with compound C (10 μM) or DMSO for 30 min prior to addition of genistein (100 μM), resveratrol (75 μM), AICAR (0.5mM), A-769662 (50 μM) or DMSO. Total RNA was isolated for qPCR to assess SHP and PPIA mRNA expression levels after a 4 h drug treatment. The SHP mRNA levels were normalized to PPIA mRNA levels. For each of three independent biological experiments, triplicate samples were used for each treatment and triplicate determinations were performed for each sample. For each individual experiment, the fold change was determined by comparison to the mean value obtained from cells treated with controls which was set as 1. Means and standard errors were determined from three independent biological replicates. Statistically significant differences compared to controls are depicted by the asterisks, **: $p<0.01$, ***: $p<0.001$.

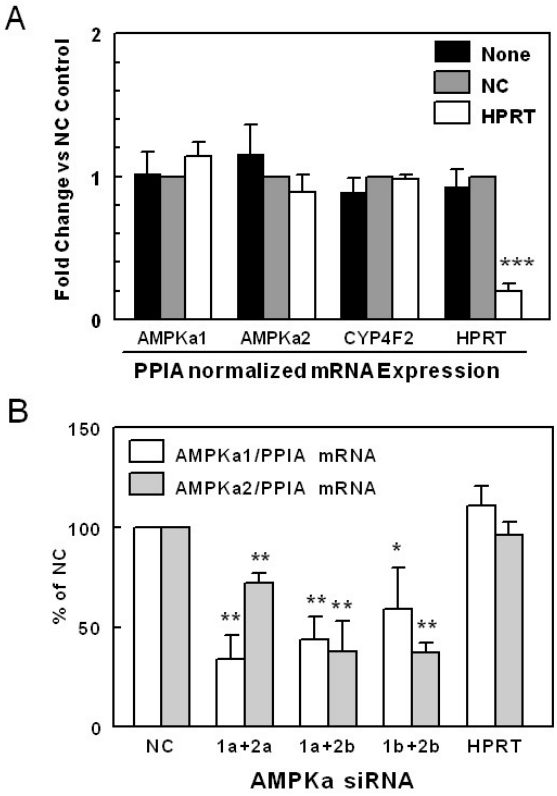

Figure S5. Effect of siRNAs on the expression of PPIA normalized mRNA expression in HepG2 cells.

(Panel A) HepG2 cells were transfected with a siRNA targeting HPRT (HPRT), a non-targeting/scrambled siRNA control (NC) or no siRNA (None) as described in "Methods". Twenty four hours later, the culture media was replaced with fresh media. Cells were harvested for total RNA isolation after another 24 h. All siRNAs were used at a final concentration of 10 nM. The PPIA normalized AMPKα1, AMPKα2, CYP4F2 and HPRT mRNA expression was expressed relative to the PPIA normalized mRNA expression in the scrambled siRNA control (NC), which is set as 1 in the same experiment. Means and standard errors were determined from at least three independent experiments. After 48 h of transfection, HPRT siRNA reduced the corresponding PPIA normalized HPRT mRNA levels by 80% relative to the scrambled siRNA controls (NC) (statistically significant at $p<0.001$,***). No significant difference in the expression of HPRT was found between mock-transfected and cells transfected with NC siRNA. No significant difference in the expression of AMPKα1, α2 and CYP4F2 was seen between the cells transfected with NC siRNA and the cells without siRNA or HPRT siRNA. (Panel B) HepG2 cells were co-transfected with both AMPKα1 (1) and AMPKα2 (2) siRNAs as described in "Methods". For each AMPKα isoform, two different siRNAs (a or b) were used. Twenty four hours later, the culture media was replaced with fresh media containing 0.5 mM AICAR or PBS. Cells were harvested for total RNA isolation after another 24 h. All siRNAs were used at a final concentration of 10 nM. The effect of AMPKα siRNA knockdown on PPIA normalized AMPKα1 and AMPKα2 mRNA expression was determined by comparing the expression of PPIA normalized AMPKα1 and AMPKα2 in the scrambled siRNA control (NC) ,which is set as 100%, in the same experiment. Means and standard errors were determined from at least three independent experiments. Statistically significant differences between NC and AMPK or HPRT targeting siRNAs are indicated as: *, $p<0.05$; **, $p<0.01$.

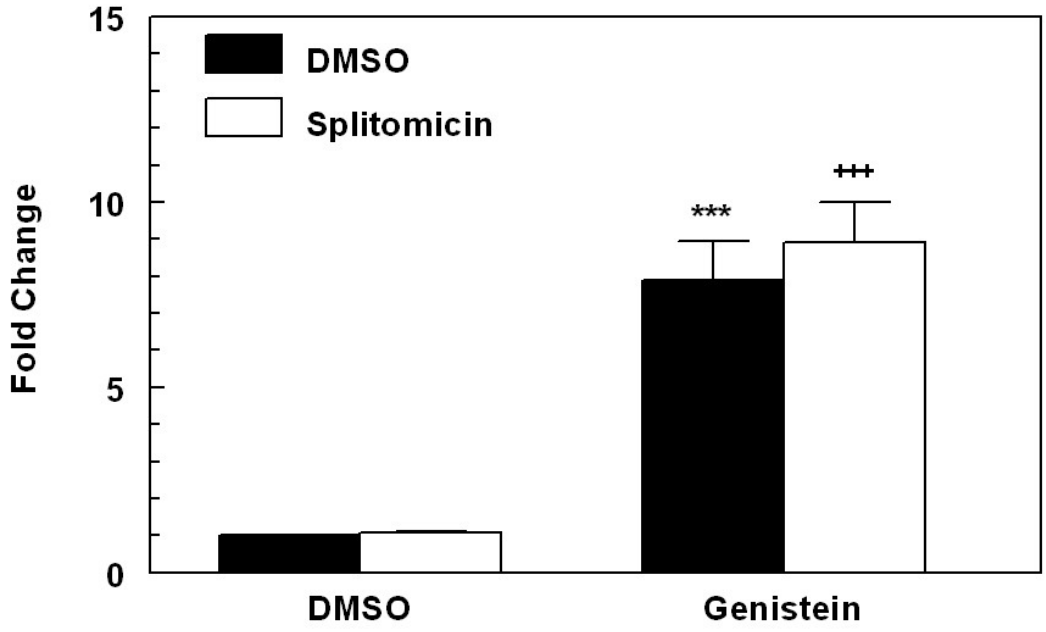

Figure S6. Stimulation of PPIA normalized *CYP4F2* mRNA expression by genistein is independent of SIRT1 activation.

HepG2 cells were pretreated with 100 μM splitomicin (a SIRT1 inhibitor) for 24 h, and then 100 μM genistein was added to the culture medium. After a 24-h genistein treatment, the cells were harvested for total RNA isolation and qPCR analysis. The *CYP4F2* mRNA levels were normalized to PPIA mRNA levels. For each independent experiment, triplicate samples were used for each treatment and triplicate determinations were performed for each sample. For each individual experiment, the fold change was determined by comparison to the mean value obtained from the cells treated with the vehicle control which was set as 1. Means and standard errors were determined from three independent experiments. Statistically significant differences between DMSO and treatments are indicated as, ***: $p<0.001$; +++: significant difference between DMSO and genistein treatments in the presence of splitomicin, $p< 0.001$.

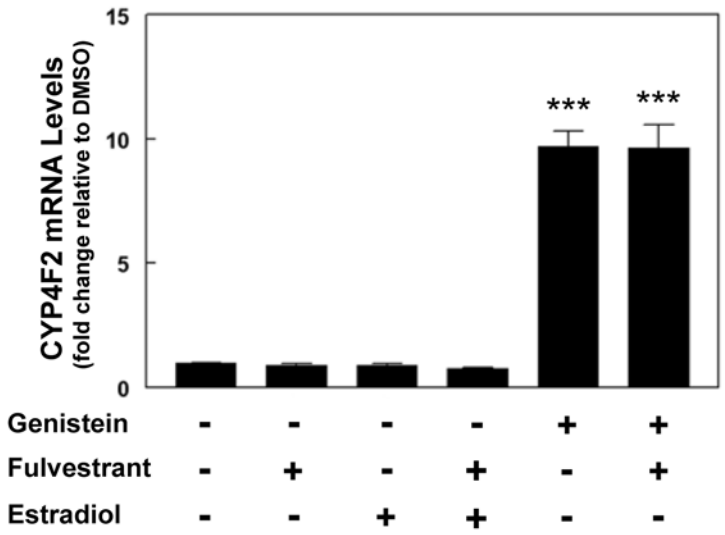

Figure S7. Genistein increases PPIA normalized *CYP4F2* mRNA expression independently of estrogen receptors. HepG2 cells were pretreated with 1 μM fulvestrant (an estrogen receptor antagonist) or DMSO for 1 h before a 24 h treatment with genistein (100 μM) or 17ß-estradiol (0.1 μM, an estrogen receptor agonist) or DMSO. Total RNA was isolated and subjected to qPCR to assess *CYP4F2* and PPIA mRNA expression levels. The *CYP4F2* mRNA levels were normalized to PPIA mRNA levels. For each independent experiment, triplicate samples were used for each treatment and triplicate determinations were performed for each sample. For each individual experiment, the fold change was determined by comparison to the mean value obtained from the cells treated with DMSO which was set as 1. Means and standard errors were determined from three independent experiments. Statistically significant differences between DMSO and drug treatments are shown, ***: $p<0.001$.

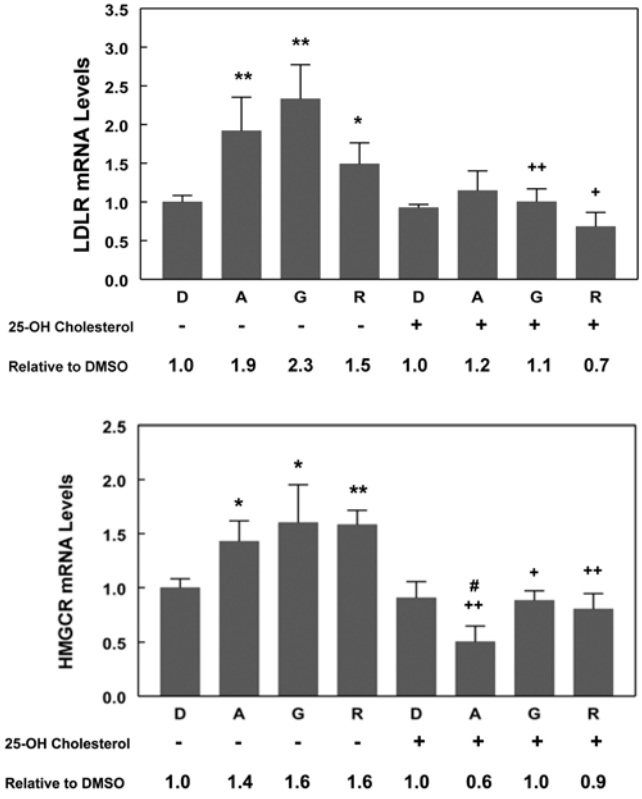

Figure S8. Effect of 25-hydroxycholesterol on SREBP target gene expression in HepG2 cells.

HepG2 cells were incubated with 25-hydroxycholesterol (25-OH cholesterol) (5 μg/ml) or vehicle control (DMSO, D) together with 0.5 mM AICAR (A), 100 μM genistein (G), or 75 μM resveratrol (R) for 24 h in culture medium containing 10% fetal bovine serum. Total RNA was isolated and qPCR employed to assess low density lipoprotein receptor (LDLR), HMG-CoA reductase (HMGCR) and PPIA mRNA expression. The LDLR (upper panel) and HMGCR (lower panel) mRNA levels were normalized to PPIA mRNA levels. For each independent experiment, triplicate samples were used for each treatment and triplicate determinations were performed for each sample. For each individual experiment, the fold change was determined by comparison to the mean value obtained from the cells treated with DMSO which was set as 1. Means and standard errors were determined from three independent experiments. Statistically significant differences between DMSO and treatments in the absence of 25-OH cholesterol, *:$p<0.05$, **: $p<0.01$; significant difference between treatments in the presence or absence of 25-OH cholesterol, +: $p<0.05$, ++: $p<0.01$; significant difference between DMSO and treatments in the presence of 25-OH cholesterol, #: $p<0.05$.