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RESEARCH ARTICLE

An ethanolic extract of *Artemisia scoparia* inhibits lipolysis in vivo and has antilipolytic effects on murine adipocytes in vitro

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[1]*Pennington Biomedical Research Center, Louisiana State University, Baton Rouge, Louisiana;* [2]*Department of Biological Sciences, Louisiana State University, Baton Rouge, Louisiana;* [3]*University of California-San Francisco, San Francisco, California; and* [4]*Department of Plant Biology and Pathology, Rutgers University, New Brunswick, New Jersey*

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Boudreau A, Richard AJ, Burrell JA, King WT, Dunn R, Schwarz JM, Ribnicky DM, Rood J, Salbaum JM, Stephens JM. An ethanolic extract of *Artemisia scoparia* inhibits lipolysis in vivo and has antilipolytic effects on murine adipocytes in vitro. *Am J Physiol Endocrinol Metab* 315: E1053–E1061, 2018. First published August 28, 2018; doi:10.1152/ajpendo.00177.2018.—An ethanolic extract of *Artemisia scoparia* (SCO) has metabolically favorable effects on adipocyte development and function in vitro and in vivo. In diet-induced obese mice, SCO supplementation significantly reduced fasting glucose and insulin levels. Given the importance of adipocyte lipolysis in metabolic health, we hypothesized that SCO modulates lipolysis in vitro and in vivo. Free fatty acids and glycerol were measured in the sera of mice fed a high-fat diet with or without SCO supplementation. In cultured 3T3-L1 adipocytes, the effects of SCO on lipolysis were assessed by measuring glycerol and free fatty acid release. Microarray analysis, qPCR, and immunoblotting were used to assess gene expression and protein abundance. We found that SCO supplementation of a high-fat diet in mice substantially reduces circulating glycerol and free fatty acid levels, and we observed a cell-autonomous effect of SCO to significantly attenuate tumor necrosis factor-α (TNFα)-induced lipolysis in cultured adipocytes. Although several prolipolytic and antilipolytic genes were identified by microarray analysis of subcutaneous and visceral adipose tissue from SCO-fed mice, regulation of these genes did not consistently correlate with SCO's ability to reduce lipolytic metabolites in sera or cell culture media. However, in the presence of TNFα in cultured adipocytes, SCO induced antilipolytic changes in phosphorylation of hormone-sensitive lipase and perilipin. Together, these data suggest that the antilipolytic effects of SCO on adipose tissue play a role in the ability of this botanical extract to improve whole body metabolic parameters and support its use as a dietary supplement to promote metabolic resiliency.

adipocyte; *Artemisia scoparia*; botanical; lipolysis

INTRODUCTION

Once regarded simply as a storage site for excess energy, adipose tissue is now known to be a major regulator of metabolic health (32). Disruption of adipose tissue's ability to store lipid or to respond to insulin, or dysregulation of its endocrine functions, have all been implicated in the metabolic dysfunction that accompanies obesity and type 2 diabetes (64). Adipose tissue lipolysis, the process by which adipocytes release fatty acids and glycerol into the circulation, occurs in response to a variety of stimuli, including fasting. Insulin resistance and obesity are often associated with abnormally high rates of basal lipolysis in the fed state, resulting in elevated circulating fatty acid levels that can further promote insulin resistance and impair metabolic functions in several tissues (41).

Plants have a long history of medicinal use in many cultures, and many modern pharmaceuticals have been developed from botanical sources. Screening efforts in our laboratory identified an ethanolic extract of *Artemisia scoparia* (SCO) that promotes adipocyte development in vitro (49). Subsequent studies revealed that SCO is a highly specific activator of peroxisome proliferator-activated receptor-γ (PPARγ) but not of any other nuclear receptor (47). In vivo, SCO, administered by oral gavage or through diet supplementation, exerts metabolically beneficial effects such as improved whole body insulin sensitivity and reduced circulating triglycerides and adiponectin, as well as many favorable effects on adipose tissue, including enhanced endocrine function and insulin signaling, and reduced monocyte chemoattractant protein-1 (MCP-1) levels, while producing no changes in body weight, food intake, body composition (47, 49, 65), fat pad weight independent of depot (observed, data not published), or de novo lipogenesis in liver or adipose tissue (52). In vitro, experiments in 3T3-L1 cells have also demonstrated SCO's ability to enhance adipogenic differentiation and to reduce tumor necrosis factor-α (TNFα)-induced changes in inflammatory gene expression and adipokine secretion (49). Given the important role of lipolysis in insulin resistance and metabolic health (41), we hypothesized that SCO might be able to modulate adipocyte lipolysis.

MATERIALS AND METHODS

Animals and extract preparation. SCO was grown under common greenhouse conditions at Rutgers University and harvested as the total plant above soil level during late-stage flowering, when seeds are beginning to develop. The ethanolic extract used for diet formulation and cell culture experiments was prepared as previously reported (49). C57BL/6J diet-induced obese (DIO) mice were purchased from Jackson Laboratories (Bar Harbor, ME), housed, randomized, and fed as described (in Ref. 49) with Research Diets [New Brunswick, NJ; high-fat diet (HFD) D12492 (60% kcal as fat)] with or without 1%

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wt/wt SCO supplementation. Mice had ad libitum access to assigned diets and water for 4 wk. Two separate cohorts of mice from this feeding study were used. Treatment of the two cohorts differed only at the end of study on *day 28/29* of defined diet feeding. For *cohort 1*, which was used for determination of circulating glycerol and fatty acids, on *day 29*, food was removed between 1200 and 1745, and lights were turned off at 1800. At 2200, animals were euthanized, and blood was collected. Blood samples were centrifuged at 3,500 rpm for 15 min, and the serum was separated, aliquoted, and frozen at −80°C until time of analysis. For *cohort 2*, which was used for gene and protein expression analyses of adipose tissue (by microarray, qPCR, and immunoblotting), animals were euthanized on *day 28*, after a 4-h fast starting at 0630. All animal studies were approved by the Pennington Biomedical Research Center Institutional Animal Care and Use Committee (Protocol no. 665P).

Serum fatty acids and glycerol. Serum lipids were extracted using 2:1 chloroform-methanol based on the Folch method (12). After total lipid extraction, the chloroform layer was dried under nitrogen and resuspended in 1 ml of chloroform and 1 mL of 3N methanolic HCl. Tubes were incubated at 37°C overnight. After incubation, 2 ml of 5% NaCl and 3 ml of hexane were added to the tubes and vortexed. The top layer was used for fatty acid analysis, and the lower layer was used for glycerol analysis. The nonaqueous layer containing the fatty acids was dried under nitrogen and resuspended in hexane for GC-MS analysis. The aqueous phase containing the glycerol was processed using a series of cation and anion columns and then dried using a Speed Vac. The sample was then derivatized using 100 μl of pyridine: acetic anhydride (1:2). Samples were heated at 60°C for 30 min, dried under nitrogen, and then resuspended in ethyl acetate for analysis by GC-MS (Agilent, Santa Clara, CA) (52).

Cell culture and lipolysis measurements. Murine 3T3-L1 preadipocytes were grown and differentiated as previously described (6). Fully differentiated 3T3-L1 adipocytes were pretreated in their regular medium [Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO) plus 10% fetal bovine serum (FBS; Hyclone, GE Life Sciences, Logan, UT)] with 50 μg/ml SCO for 72–96 h and 0.5–0.75 nM TNFα (Life Technologies, Carlsbad, CA) for 16 h, or respective vehicle controls. Stock solutions were at 50 mg/ml in DMSO for SCO, and 0.5 μM in phosphate-buffered saline (PBS) plus 0.1% bovine serum albumin (BSA, Sigma-Aldrich) for TNFα. In each case, equal volumes of stock solutions and their respective vehicles were used. After pretreatment, medium was removed, and cells were incubated in phenol red-free DMEM (Life Technologies) containing 0.1% glucose and 2% BSA for 2.5 h. Conditioned media were collected and assayed for glycerol by use of free glycerol reagent (Sigma-Aldrich), or for nonesterified fatty acids (NEFA) by use of a free fatty acid (FFA) quantification kit (BioVision, Milpitas, CA). Absorbances were measured on a Versa Max spectrophotometer using Spectromax software (Molecular Devices, Sunnyvale, CA).

Microarray analysis of gene expression. Inguinal (iWAT) and epididymal (eWAT) adipose tissue depots from mice fed a HFD with or without SCO supplementation for 4 wk (as described above) were harvested and flash-frozen. RNA was then purified using the RNeasy mini kit (Qiagen, Hilden, Germany), and reverse transcribed using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). Gene expression analysis of SCO-exposed compared with nonexposed samples was performed as described previously (26), using Illumina mouse expression arrays (Illumina, San Diego, CA) and following protocols specified by the manufacturer. Briefly, raw array data were extracted and processed by Limma software (51), which included background subtraction, identification of expressed genes, quantile normalization, and log2 transformation. Differentially expressed genes were identified through a Bayesian-moderated *t*-test (yielding Bayes-regularized *P* values), as implemented in CyberT software (29). Data were deposited to the Gene Expression Omnibus (GEO) database (accession no. GSE113808). Results were examined for effects of SCO on a list of 36 genes selected on the basis of literature searches for their involvement in lipolysis (shown in Table 1). Genes with *P* values below 0.05 were considered differentially expressed.

Quantitative polymerase chain reaction gene expression analysis. A subset of SCO-regulated genes identified in iWAT and eWAT by microarray analysis was validated by quantitative polymerase chain reaction (qPCR) analysis using RNA prepared as described above. For in vitro studies, cells were harvested, and RNA was isolated using the RNeasy mini kit. Reverse transcription was performed with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Takara SYBR premix (Takara Bio USA, Mountain View, CA) and primers from IDT (Integrated DNA Technologies, Skokie, IL) were used to perform qPCR on the Applied Biosystems 7900 HT system with SDS 2.4 software (Applied Biosystems). Thermal cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C; dissociation stage: 15 s at 95°C, 15 s at 60°C, and 15 s at 95°C. *Nono* (non-POU domain containing octamer-binding protein) and *Ppia* (peptidylprolyl isomerase A) were used as reference genes. Primer sequences are shown in Table 2.

Whole cell extract preparation. Adipocyte monolayers from experiments described above were harvested in a buffer containing 150 mM NaCl, 10 mM Tris (pH 7.4), 1 M ethylene glycol tetraacetic acid (EGTA), 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100; 0.5% Igepal CA-630, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 50 trypsin inhibitory milliunits of aprotinin, 10 μM leupeptin, 1 mM 10-phenanthroline, and 0.2 mM sodium orthovana-

Table 1. *List of lipolysis-related genes used to query microarray data from SCO feeding study*

Gene Name	Gene Symbol	References*
Abhydrolase domain containing 5	*Abhd5*	(13)
Adenosine A1 receptor	*Adora1*	(7)
Adenosine A2b receptor	*Adora2b*	(7)
Adrenoceptor alpha 1A	*Adra1a*	(13)
Adrenoceptor alpha 1B	*Adra1b*	(13)
Adrenoceptor alpha 2A	*Adra2a*	(13)
Adrenoceptor alpha 2B	*Adra2b*	(13)
Adrenoceptor alpha 2C	*Adra2c*	(13)
Adrenoceptor beta 1	*Adrb1*	(9, 18)
Adrenoceptor beta 2	*Adrb2*	(9, 18)
Adrenoceptor beta 3	*Adrb3*	(9, 18)
Aquaporin 7	*Aqp7*	(13)
Arrestin beta 1	*Arrb1*	(9, 28)
Caveolin 1	*Cav1*	(13)
Endothelin 1	*Edn1*	(25)
Free fatty acid receptor 2	*Ffar2*	(15)
G0/G1 switch 2	*G0s2*	(23)
Hydroxycarboxylic acid receptor 2	*Hcar2*	(69)
Leptin	*Lep*	(19)
Lipase, hormone sensitive	*Lipe*	(9, 31)
Mitogen-activated protein kinase kinase kinase 8	*Map3k8*	(21)
Melanocortin 2 receptor	*Mc2r*	(40)
Melanocortin 5 receptor	*Mc5r*	(40)
Monoglyceride lipase	*Mgll*	(13)
Natriuretic peptide receptor 1	*Npr1*	(7)
Natriuretic peptide receptor 2	*Npr2*	(7)
Natriuretic peptide receptor 3	*Npr3*	(7)
OPA1, mitochondrial dynamin like gtpase	*Opa1*	(10)
Phosphodiesterase 3b	*Pde3b*	(9, 45, 68)
Phosphodiesterase 4a	*Pde4a*	(34)
Phosphodiesterase 4b	*Pde4b*	(34)
Phosphodiesterase 4d	*Pde4d*	(34)
Phosphodiesterase 5	*Pde5*	(3)
Perilipin	*Plin*	(9, 53)
Patatin-like phospholipase domain containing 2	*Pnpla2*	(9, 31)
Succinate receptor 1	*Sucnr1*	(38)

SCO, *Artemisia scoparia*. *References that refer to a gene as playing a role in lipolysis.

Table 2. *Primer sequences for quantitative PCR gene expression analysis*

Gene Name (Symbol)	Forward Primer, 5′-3′	Reverse Primer, 5′-3′
Cyclophilin A (*Ppia*)	CCACTGTCGCTTTTCGCCGC	TGCAAACAGCTCGAAGGAGACGC
Non-POU domain containing octamer binding protein (*Nono*)	CATCATCAGCATCACCACCA	TCTTCAGGTCAATAGTCAAGCC
Perilipin 1 (*Plin1*)	CGTGGAGAGTAAGGATGTCAATG	GTGCTGTTGTAGGTCTTCTGG
Phosphodiesterase 3b (*Pde3b*)	GTCGTTGCCTTGTATTTCCC	CAACTCCATTTCCACCTCCA
G0/G1 switch 2 (*G0s2*)	CAAAGCCAGTCTGACGCAA	CCTGCACACTTTCCATCTGA
Hormone-sensitive lipase (*Lipe*)	CTGCAAGAGTATGTCACGCTA	CTCGTTGCGTTTGTAGTGC
Patatin like phospholipase domain containing 2 (*Pnpla2/Atgl*)	GAGCTCATCCAGGCCAAT	CTCATAAAGTGGCAAGTTGTCTG
Adrenergic receptor, beta 3 (*Adrb3*)	CCACCGCTCAACAGGTTT	CCAGAAGTCCTGCAAAAACG

date. Lysates were subjected to one freeze-thaw cycle, passed through a 20-gauge needle three times, and then centrifuged at 17,500 *g* at 4°C for 10 min. Supernatants were recovered, and protein content was measured by bicinchoninic acid (BCA) protein assay (Sigma-Aldrich).

Gel electrophoresis and immunoblotting. Protein (100 μg/well) was loaded onto 10% polyacrylamide gels and transferred to nitrocellulose membranes, which were probed and imaged using standard immunoblotting techniques and detected using a horseradish peroxidase-conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA) and SuperSignal West Pico PLUS detection reagents (Thermo Scientific, Rockford, IL). Polyclonal rabbit antibodies against hormone-sensitive lipase (HSL), phospho-HSL (Ser^{563}, Ser^{565}, or Ser^{660}), and perilipin, as well as a mouse monoclonal antibody against β-actin, were obtained from Cell Signaling Technology (Danvers, MA; catalog nos. 4107, 4139, 4137, 4126, 3470, and 3700, respectively). A mouse monoclonal antibody against phospho-perilipin (human Ser^{522}/mouse Ser^{517}) was obtained from Vala Sciences (San Diego, CA; catalog no. 4856). Autoradiography films were scanned, and densitometry was performed using Image Studio software (Li-Cor Biosciences, Lincoln, NE).

Statistics. GraphPad Prism 6 for Windows software (La Jolla, CA) was used to calculate means and SE, produce graphs, and determine statistical significance using unpaired two-tailed *t*-tests or two-way ANOVA with Tukey's multiple comparisons test. Threshold for significance was set at $P < 0.05$.

RESULTS

On the basis of our previous studies, we hypothesized that SCO might affect another important metabolic pathway in adipose tissue, namely lipolysis. First, we examined the levels of FFAs and glycerol in serum of mice fed HFD (60% kcal as fat) for 4 wk in the presence or absence of 1% (wt/wt) of SCO extract. As shown in Fig. 1, supplementation with SCO significantly reduced the circulating levels of glycerol and of all fatty acids measured (C18:0, C18:1, C16:0, C16:1), suggesting that SCO may have direct effects on adipose tissue to reduce lipolysis.

The relationships between inflammation, lipolysis, and insulin resistance (11, 27, 44) prompted us to examine whether SCO could affect lipolysis under basal and induced conditions in a cell-autonomous manner in adipocytes. We stimulated lipolysis either with TNFα or by β-adrenergic stimulation with isoproterenol in murine 3T3-L1 adipocytes. As shown in Fig. 2, TNFα and isoproterenol strongly induced lipolysis, as measured by both glycerol and FFA release into the culture medium. Chronic pretreatment (72 h) with SCO had no significant effects on basal or isoproterenol-induced lipolysis, but significantly attenuated the effect of TNFα.

To investigate the potential mechanisms by which SCO may be modulating lipolysis, we performed microarray analysis on visceral eWAT and subcutaneous iWAT samples from male mice fed a HFD with or without SCO supplementation. The resulting data were examined for effects of SCO on 36 genes known to be involved in lipolysis. The list of genes queried is shown in Table 1; genes from this list found to be regulated by SCO ($P < 0.05$; 10 in iWAT, 8 in eWAT), and their corresponding fold changes, are shown in Table 3. Expression of some of these genes was regulated in a manner that would be expected to reduce lipolysis. For example, *Lipe/Hsl* and *Adrb3* (β_3-adrenergic receptor) are both downregulated in iWAT and eWAT, and *Pde3b* is downregulated in iWAT. However, the effect of SCO on other genes could be considered prolipolytic. One example is the reduced expression of *G0s2* in iWAT. A subset of the SCO-regulated genes was analyzed by qPCR to validate the microarray results (Fig. 3). All genes evaluated were regulated in the same direction as observed in the microarray analysis, although not all of the SCO-induced changes were statistically significant when analyzed by qPCR.

Stimulation of lipolysis by TNFα is associated with the inhibition of several antilipolytic genes including *Plin*, *Pde3b*, and *G0s2* (23, 45, 67). Hence, we measured the effects of TNFα and SCO on the expression of these genes in 3T3-L1 adipocytes. As shown in Fig. 4, we observed the expected reductions in expression with TNFα treatment. However, SCO did not reverse these changes, suggesting that the effect of SCO on TNFα-mediated lipolysis is likely independent of the transcriptional regulation of these genes. Although TNFα pro-

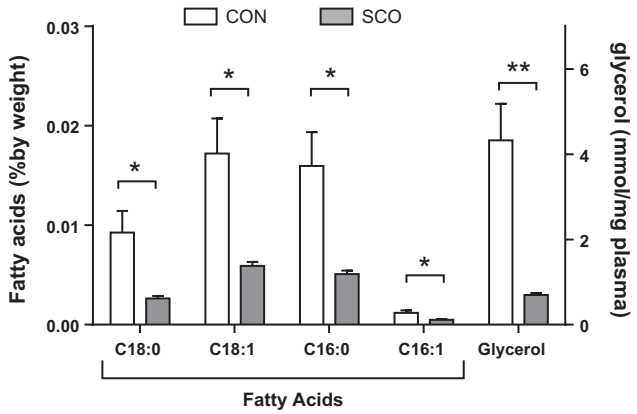

Fig. 1. *Artemisia scoparia* (SCO) supplementation in high-fat diet (HFD) reduces circulating levels of free fatty acids and glycerol. C57BL/6 diet-induced obese (DIO) mice were fed a HFD supplemented with 1% wt/wt SCO (SCO) or without (CON) ad libitum for 4 wk. Mice were fasted for 4 h, food was returned to the cages, and mice were euthanized 4 h later. Serum lipids and glycerol were extracted and analyzed by GC-MS. Data are presented as means ± SE ($n = 5$ mice per group). $^*P < 0.05$, $^{**}P < 0.01$.

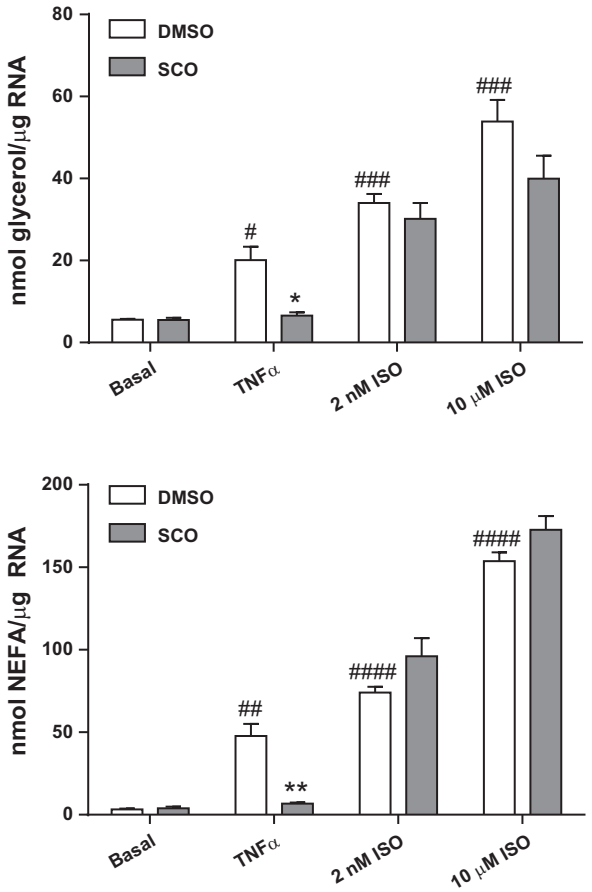

Fig. 2. *Artemisia scoparia* (SCO) reduces tumor necrosis factor-α (TNFα)-induced, but not isoproterenol (ISO)-induced or basal lipolysis in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes, cultured in 6-well plates, were pretreated for 3 days with SCO at 50 μg/ml and overnight with 0.75 nM TNFα or equal volumes of their vehicles (DMSO or 0.1% BSA in PBS, respectively). Culture medium was replaced with lipolysis incubation medium containing 0 or 2 nM or 10 μM ISO. Medium was assayed for glycerol and nonesterified fatty acid (NEFA) concentrations after 2.5 h, and resulting concentrations were corrected for total RNA measured in harvested cells. Data are displayed as means ± SE; $n = 3$ replicate cell culture wells per condition. Significance denoted as $^{*}P < 0.05$ and $^{**}P < 0.01$ (for SCO treatment vs. DMSO control); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$ (for TNFα or ISO treatment vs. basal). Data shown are representative of an experiment that was repeated 2 (for the ISO treatments) or 3 times (for the TNFα treatment).

duces robust activation of lipolysis, it has also been shown to reduce the expression of genes coding for proteins that promote lipolysis, such as *Adrb3*, *Lipe*/*Hsl*, and *Pnpla2*/*Atgl* (18, 23, 31). In our experiments, TNFα treatment of 3T3-L1 adipocytes produced the expected reductions in expression of these genes, but the only significant effects of SCO on these genes were reductions in *Atgl* and *Adrb3* in the absence of TNFα. Although these effects would be considered antilipolytic, they cannot explain the effect of SCO on lipolysis, since SCO had no effect on lipolysis in the absence of TNFα. Moreover, reduction in adrenergic signaling would also be expected to reduce isoproterenol-induced lipolysis, which did not occur with SCO treatment. Conversely, in the presence of TNFα, where SCO does reduce lipolysis it had no effect on *Atgl* or *Adrb3* expression (Fig. 4).

Since lipolysis rates are largely determined by posttranscriptional and posttranslational regulation of lipolytic and lipid droplet-associated proteins (reviewed in Ref. 13), we examined the effects of SCO on perilipin and HSL protein levels and phosphorylation. Consistent with our gene expression studies (Figs. 3 and 4), we observed that TNFα reduced HSL protein levels and that SCO did not significantly modulate HSL levels (Fig. 5*A*). However, SCO treatment was associated with altered phosphorylation of two serine residues (Ser^{563} and Ser^{660}) in HSL known to increase the enzyme's activity and lipolysis rates (1, 16, 56). Although TNFα reduced total HSL levels, it did not alter the amount of HSL phosphorylated at Ser^{563} (HSL $pSer^{563}$). These observations are consistent with reported actions of TNFα to increase both HSL phosphorylation and lipolysis despite reductions in total HSL levels (36, 59, 68, 70). Although SCO had no effect on HSL $pSer^{563}$ in unstimulated conditions, it reduced HSL $pSer^{563}$ in the presence of TNFα. A similar pattern was observed with the other prolipolytic phosphorylation site that we examined, HSL $pSer^{660}$ (Fig. 5, *A* and *B*). We also studied the phosphorylation of HSL at Ser^{565}, which inhibits phosphorylation of HSL at Ser^{563} and is associated with decreased HSL activity (2, 14, 30). TNFα produced a significant reduction in the amount of HSL $pSer^{565}$, but we did not observe any modulation by SCO. Perilipin is a major regulator of lipolysis, as it controls access of HSL to the lipid droplet, and its phosphorylation is a potent inducer of lipolysis (39, 63). As shown in the bottom panels of Fig. 5*A* and *C*, SCO had no effect on total perilipin protein levels but produced a striking reduction in its phosphorylation at Ser^{517}, a prolipolytic modification. In Fig. 5, *B* and *C*, bands shown in Fig. 5*A* were quantitated by densitometry, and normalized to the loading control, β-actin.

DISCUSSION

An ethanolic extract of *A. scoparia* (SCO) was originally identified in a screen of botanical extracts that modulated

Table 3. *SCO supplementation in high-fat diet significantly modulates the expression of several lipolysis-related genes*

iWAT			eWAT		
Gene	FC	*P* Value	Gene	FC	*P* Value
		Antilipolytic regulation			
Adrb3	−2.83	7.58E-05	*Mgll*	−2.15	1.26E-03
Aqp7	−3.28	1.05E-04	*Adrb3*	−2.16	0.022
Lipe	−2.11	1.58E-03	*Lipe*	−1.83	0.041
Pnpla2	−1.36	5.60E-03	*Arrb1*	−1.41	0.045
Pde4a	1.33	0.014			
Pde3b	1.35	0.031			
		Prolipolytic regulation			
Plin	−1.66	0.014	*Adcy9*	−1.96	3.42E-03
Npr3	2.70	2.77E-03	*Adrb2*	1.37	0.034
G0s2	−1.85	2.81E-03	*Pde4b*	−1.28	0.043
Map3k8	2.37	3.65E-03			

Diet-induced obese (DIO) mice were fed a high-fat diet with or without supplementation with 1% wt/wt *Artemisia scoparia* (SCO) for 4 wk ($n = 4$ mice per group). Mice were euthanized, and adipose tissue depots were harvested and flash-frozen. RNA was purified, and gene expression was analyzed by microarray. Results were examined for effects of SCO on 36 genes involved in lipolysis, based on literature searches. Genes significantly regulated by SCO ($P < 0.05$) in inguinal (iWAT) and epididymal (eWAT) white adipose tissue are shown along with their respective fold changes (FC) relative to controls and *P* value. Genes are separated based on whether the direction of observed change would be expected to repress (antilipolytic regulation) or induce (prolipolytic regulation) lipolysis.

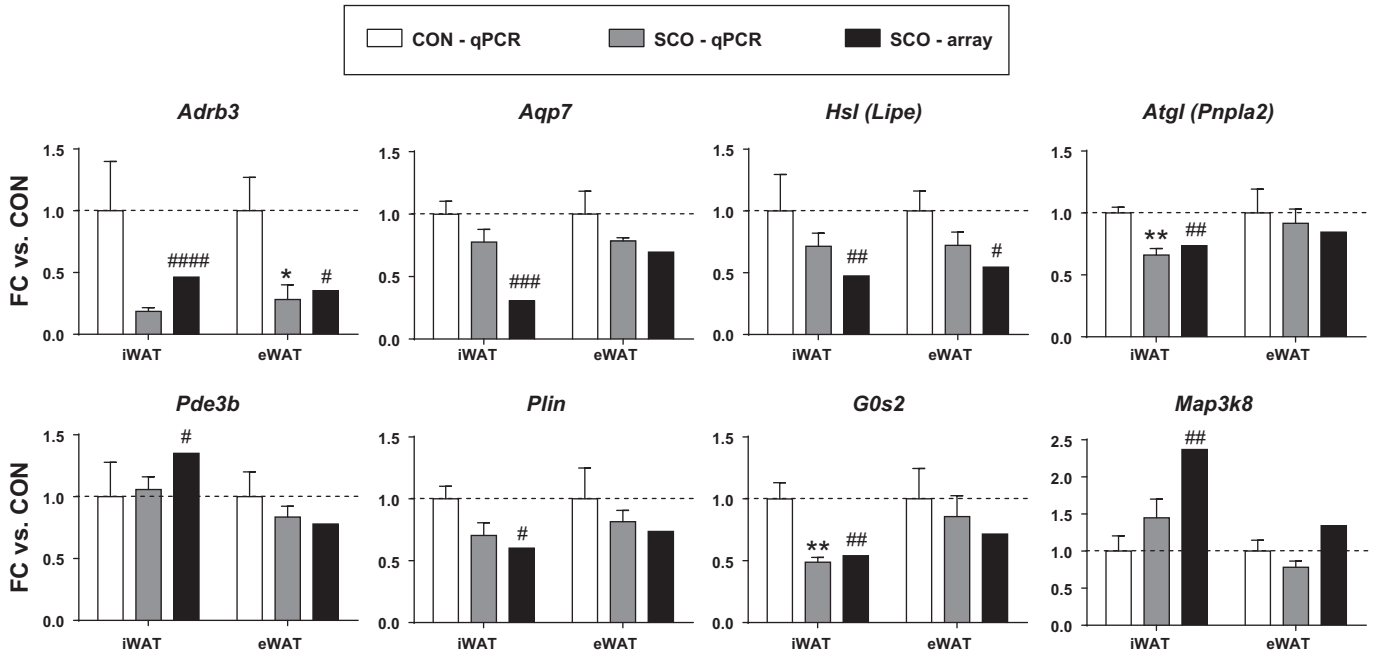

Fig. 3. qPCR validation of *Artemisia scoparia* (SCO)-regulated genes identified in microarray analysis. For validation, RNA isolated for microarray analysis was reverse transcribed, and resulting cDNA was analyzed by qPCR. Target gene data were normalized to reference genes *Ppia* and *Nono*. Data are plotted as fold change (FC) vs. control (CON); qPCR data are presented as means ± SE; $n = 5$ mice per group. Significance denoted as $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$ (for effect of SCO in microarray analysis); $^{*}P < 0.05$, $^{**}P < 0.01$ (for effect of SCO as measured by qPCR).

adipocyte development in vitro and was later shown to have metabolically beneficial effects on whole body insulin sensitivity and on adipose tissue function (47, 49). Given the important role of lipolysis in insulin resistance (22) and the favorable effects of SCO on insulin sensitivity in vivo, we hypothesized that SCO might regulate lipolysis. Our studies revealed that SCO inhibits the levels of both glycerol and FFAs (NEFAs) both in vivo and in vitro. Mice fed a HFD supplemented with SCO had significantly lower serum levels of glycerol and all the NEFAs that we examined compared with HFD controls without SCO supplementation (Fig. 1). These results suggested that SCO likely reduces lipolysis in adipose tissue and might affect adipocytes directly. In addition to these in vivo observations, we found that SCO attenuated TNFα-induced lipolysis but did not substantially affect basal or isoproterenol-induced release of glycerol and NEFAs in cultured 3T3-L1 adipocytes (Fig. 2).

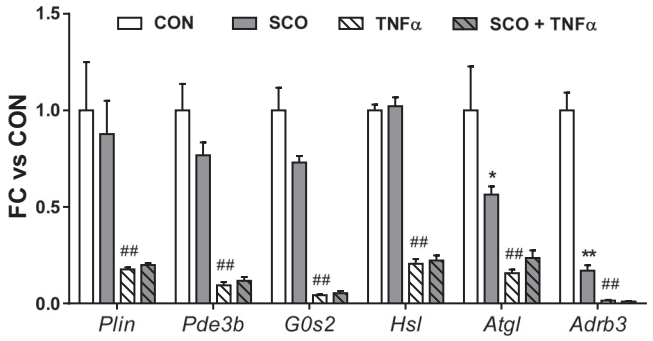

Fig. 4. *Artemisia scoparia* (SCO) does not reverse tumor necrosis factor-α (TNFα)-induced changes in the expression of genes involved in lipolysis. Differentiated 3T3-L1 adipocytes were pretreated for 3–4 days with SCO at 50 μg/ml and/or 0.5 to 0.75 nM TNFα or an equal volume of their respective vehicles (DMSO or 0.1% BSA in PBS). Control (CON) wells were treated with both vehicles. Cells were harvested, and RNA was isolated, reverse transcribed, and subjected to qPCR. Target gene data were normalized to the reference gene *Nono*. Data are displayed as means ± SE; $n = 3$ replicate cell culture wells per condition. Significance denoted as $^{*}P < 0.01$, $^{**}P < 0.0001$ (for SCO treatment vs. CON); $^{\#\#}P < 0.0001$ (for TNFα treatment vs. CON). These data are representative of an experiment performed in duplicate.

The association of TNFα and adipose tissue inflammation is well documented. TNFα not only promotes insulin resistance by decreasing the expression of GLUT4 (61), the insulin receptor, and IRS-1 (60) but also is a potent stimulator of lipolysis (27). In conditions of obesity and insulin resistance, TNFα can be produced in adipose tissue macrophages and act in a paracrine manner on adjacent adipocytes to promote metabolic dysfunction, enhance basal lipolysis, and raise circulating fatty acid levels (11). Increased TNFα and circulating levels of FFAs and their association with insulin resistance have also been documented in a clinical study (44). Hence, the ability of SCO to attenuate TNFα-stimulated lipolysis has relevance in the context of obesity and Type 2 diabetes.

To investigate the potential mechanisms involved in the ability of SCO to regulate lipolysis, we performed microarray analysis and examined the expression of lipolysis-related genes (Table 1) in both subcutaneous and visceral adipose tissue samples from an animal study where mice were fed SCO for 4 wk in the presence of a HFD. This analysis yielded equivocal results. Specifically, few genes were significantly regulated by SCO; however, not all genes that were altered by SCO were changed in a manner expected to reduce lipolysis (Table 3). There are several factors that could account for our observations. First, lipolysis rates are highly regulated at the protein level through mechanisms such as phosphorylation and protein stability (43). Although we validated our array results with qPCR (Fig. 3), it is known that gene expression is not always

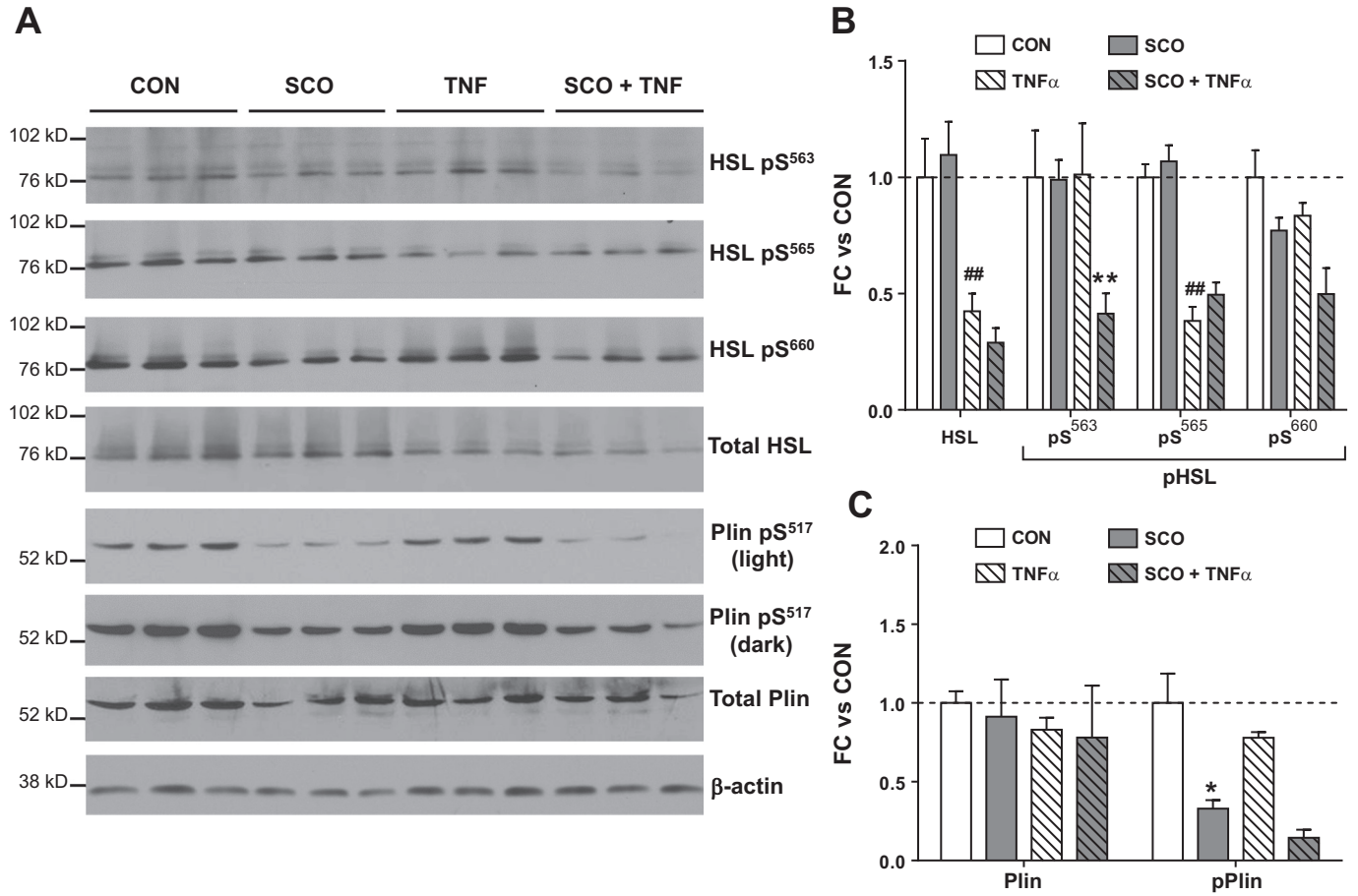

Fig. 5. *Artemisia scoparia* (SCO) induces antilipolytic changes in phosphorylation of hormone-sensitive lipase (HSL) and perilipin. Differentiated 3T3-L1 adipocytes were pretreated for 3 days with SCO at 50 μg/ml and overnight with 0.75 nM tumor necrosis factor-α (TNFα) or equal volumes of their vehicles (DMSO or 0.1% BSA in PBS, respectively). Control (CON) wells were treated with both vehicles. Culture medium was replaced with lipolysis incubation medium for 2.5 h for glycerol and nonesterified fatty acid (NEFA) measurements, and then cells were harvested and whole cell extracts were prepared; 100 μg total protein per well were loaded for Western blot analysis. Blot images are shown in *A*. The HSL Ser^{563} and Ser^{660} phosphorylation sites are associated with enhanced lipolysis, whereas phosphorylation at Ser^{565} is antilipolytic. *B* and *C*: band intensities were quantified by densitometry. All bands were normalized to the loading control (β−actin). Data are expressed as means ± SE fold change (FC) vs. CON (basal conditions). Significance denoted as $^{\#\#}P < 0.01$ (for effect of TNFα vs. CON), and $^{*}P < 0.05$ and $^{**}P < 0.01$ (for effect of SCO vs CON or TNFα only); $n = 3$ replicate wells per condition.

reflective of protein levels or activity. Another consideration is that, although adipocytes are the prominent cell type found in adipose tissue, fat tissue is composed of many other cell types (preadipocytes, immune cells such as lymphocytes and macrophages, and others). Therefore, the gene expression measured in adipose tissue samples reflects mRNA levels in all adipose tissue cell types. The effects of SCO on gene expression in nonadipocytes may account for some of our microarray observations (Table 3 and Fig. 3). Of the lipolytic genes that we examined in cultured adipocytes, only *Adrb3* was modulated by SCO, but only in the absence of TNFα, a condition in which we did not observe effects of SCO on lipolysis.

In cultured adipocytes, TNFα affects the expression and posttranslational modifications of proteins known to regulate lipolysis (reviewed in Refs. 13, 55). Hence, we also examined the effects of TNFα and SCO on HSL and perilipin expression and phosphorylation (Fig. 5). As previously documented, TNFα reduced HSL levels (59) even though it enhanced lipolysis. SCO did not significantly modulate HSL levels but did alter the phosphorylation of two serine residues in HSL known to increase the enzyme's activity and lipolysis rates (1, 16, 56). Although TNFα reduced total HSL levels, it did not affect phosphorylation of HSL Ser^{563}. Our studies are consistent with evidence that TNFα increases HSL phosphorylation and lipolysis despite its ability to reduce total HSL levels (36, 59, 68, 70). While SCO alone had no effect on HSL pS^{563} in basal conditions, phosphorylation at this residue was reduced in the presence of TNFα. A similar pattern was observed with the other prolipolytic phosphorylation site, HSL Ser^{660}. The phosphorylation of HSL at Ser^{565} inhibits activation of HSL and is associated with decreased HSL activity (2, 14, 30). Although TNFα produced a significant reduction in the amount of HSL $pSer^{565}$, we did not observe any modulation by SCO. In terms of HSL, our studies suggest that SCO does not impact HSL protein levels but can reduce the TNFα-induced activation of the prolipolytic phosphorylation at Ser^{563} and Ser^{660}. Perilipin regulates lipolysis by controlling access of HSL to the lipid droplet, and its phosphorylation strongly induces lipolysis (39, 63). Our studies demonstrated that SCO had no effect on total perilipin protein levels but produced a striking reduction in its prolipolytic phosphorylation at Ser^{517} (Fig. 5, *A* and *C*). Phosphorylation at this site has also been shown to be impor-

tant in human perilipin at Ser^{522} (62). Clearly, SCO treatment could affect the ability of TNFα to regulate lipolysis by modulating the phosphorylation of known serine residues in HSL and perilipin. Previous work from our laboratory has shown that SCO activates the nuclear receptor PPARγ and modulates expression of its target genes, which, in turn, enhance adipocyte differentiation (47, 49). Effects of SCO on lipolysis presented in our current study appear to be largely mediated by posttranslational events rather than at the transcriptional level. Interestingly, inhibition of PPARγ has been shown to be involved in mediating TNFα's ability to stimulate lipolysis, and PPARγ agonists inhibit TNFα-induced lipolysis (23, 57, 59). In addition, the ability of pioglitazone (a PPARγ agonist) to inhibit TNFα's effect on lipolysis is independent of PPARγ's adipogenic activity (20). This raises the possibility that SCO's effects on adipogenesis and on TNFα-induced lipolysis may both be dependent on PPARγ activation.

Recently, SCO has been reported to have anti-inflammatory effects in human mast cells by reducing the levels of TNFα and other inflammatory cytokines (42). These studies also showed that SCO and one of its constituents, 3,5-dicaffeoyl-epi-quinic acid, could reduce the nuclear translocation of nuclear factor-κB (NF-κB). Our studies revealed that SCO attenuated only TNFα-induced lipolysis and not basal or adrenergic-induced lipolysis in cultured adipocytes (Fig. 2). Various mechanisms have been implicated in the ability of TNFα to induce lipolysis, including impairment of insulin signaling (9, 33), repression of the antilipolytic protein G0S2 (23), modulation of the micro-RNA miR-145 (37), and the induction of extracellular signal-regulated kinase (ERK) (57), as well as the regulation of cell death-inducing DFFA-like effector c, also known as fat-specific protein-27 (CIDEC/FSP27) (46). Our measurements of circulating glycerol and fatty acids were made in the fed state, when insulin levels are high. Given that insulin is a potent inhibitor of lipolysis, the insulin-sensitizing effects of SCO in vivo are likely to contribute to its effects on lipolysis; however our observation that TNFα-induced lipolysis in cultured adipocytes is reduced by SCO in the absence of insulin suggests that SCO may act, at least in part, through insulin-independent mechanisms. Related to our observations, a study in human adipocytes has shown that a peptide inhibitor of IKK (IκB kinase) activation effectively blocks the TNFα-mediated nuclear translocation of NF-κB (35). Notably, these studies also show that the ability of TNFα to induce lipolysis is dependent on NF-κB activation (35). Collectively, these reports and our results suggest that the ability of SCO to attenuate TNFα-induced lipolysis in cultured adipocytes may also be related to modulation of NF-κB activity. Our in vivo microarray analysis (Table 3), coupled with our in vitro adipocyte studies (Fig. 4), suggest that modulation of G0S2, a protein that reduces lipolysis, is not involved in the ability of SCO to reduce TNFα-induced lipolysis. Additional studies in cultured adipocytes indicate that siRNA knockdown of G0S2 did not affect the ability of SCO to regulate lipolysis in 3T3-L1 adipocytes (data not shown). Published studies by other laboratories showing that either anti-inflammatory effects of SCO (17, 42) or effects of TNFα on lipolysis are dependent on NF-κB (35) are supportive of our novel data implicating a potential role of NF-κB in the actions of SCO on adipocytes. Future studies could be performed to demonstrate a role of NF-κB in this regulation, but these studies will be challenging as loss of NF-κB expression or activation affects a variety of adipocyte signaling and inflammatory pathways. To complicate these investigations, there are likely several mechanisms involved in the ability of SCO to abrogate TNFα-induced lipolysis in adipocytes.

Our in vivo studies were performed on HFD-fed mice, in conditions that are known to promote inflammation in adipose tissue (49). It should be noted that, although we have not directly examined macrophage numbers in adipose tissue, our microarray analysis, as well as unpublished experiments, have produced no compelling evidence that SCO reduces macrophage infiltration or activation, but rather that SCO attenuates the response to inflammatory signals in adipocytes. As our in vitro experiments demonstrate, SCO treatment of cultured adipocytes renders them less responsive to TNFα, the primary macrophage-derived inflammatory mediator in adipose tissue (4, 8), and these results are consistent with an in vivo scenario in which SCO may reduce inflammatory responses in adipocytes in the absence of any effects on macrophage numbers or function. Additional studies would be needed to definitively address the question of whether SCO treatment can alter macrophage infiltration or activation in adipose tissue.

In conclusion, our studies have revealed that an extract of SCO known to have positive effects on various metabolic parameters in a mouse model of diet-induced obesity (47, 49) can also reduce serum levels of NEFAs and glycerol (Fig. 1). Lipolysis is one of several metabolically important functions of adipose tissue, and it is well known that obesity/type 2 diabetes is often associated with elevated rates of lipolysis accompanied by increased circulating levels of glycerol and FFAs (41). Notably, antilipolytic agents, such as acipimox and atglistatin, have been proposed as viable therapeutic strategies for improving insulin sensitivity and dyslipidemia (5, 24). Although our observations do not necessarily indicate a direct effect of SCO on adipose tissue lipolysis, our results support this notion and collectively indicate that the ability of SCO to promote metabolic resiliency likely occurs, at least in part, through its ability to reduce TNFα-regulated lipolysis. Direct assessment of lipolytic activity from adipose tissue explants ex vivo will be required to confirm that diet supplementation with SCO reduces lipolysis and to determine any depot-specific differences in the effects of SCO. Depot-specific differences could also potentially be assessed through in vitro treatment of primary adipocytes. Further characterization of TNFα-induced lipolysis and its reduction by SCO will also be needed to identify the mechanisms by which SCO antagonizes TNFα action. In addition, future studies should include efforts to identify the bioactive compounds present in SCO that regulate lipolysis and to elucidate other mechanisms involved in the ability of SCO to promote metabolic health. We propose that the global epidemics of obesity, type 2 diabetes, and metabolic syndrome (54) could potentially be tackled by a greater consideration of botanical-based interventions.

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DISCLAIMERS

The content in this paper is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

A.B. and J.M. Stephens conceived and designed research; A.B., J.A.B., W.T.K., R.D., J.-M.S., D.M.R., J.R., and J.M. Salbaum performed experiments; A.B., J.A.B., W.T.K., R.D., J.-M.S., J.R., and J.M. Salbaum analyzed data; A.B., A.J.R., and J.M. Stephens interpreted results of experiments; A.B., A.J.R., and J.A.B. prepared figures; A.B. and J.M. Stephens drafted manuscript; A.B., A.J.R., J.A.B., D.M.R., J.R., J.M. Salbaum, and J.M. Stephens edited and revised manuscript; A.B., A.J.R., J.A.B., W.T.K., R.D., J.-M.S., D.M.R., J.R., J.M. Salbaum, and J.M. Stephens approved final version of manuscript.

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