4-2018

Artemisia scoparia Reduces the Effects of TNFα-induced Lipolysis in Murine Adipocytes

Ruth Dunn

Artemisia scoparia Reduces the Effects of TNFα-induced Lipolysis in Murine Adipocytes

by

Ruth Dunn

Undergraduate honors thesis under the direction of

Dr. Jacqueline Stephens

Department of Biological Sciences

Submitted to the LSU Roger Hadfield Ogden Honors College in partial fulfillment of the Upper Division Honors Program.

April 2018

Louisiana State University
& Agricultural and Mechanical College
Baton Rouge, Louisiana

Abstract:

Background: Adipocytes serve a vital role in lipid storage, as well as endocrine functions and insulin sensitivity. The impairment of these actions can lead to metabolic syndrome, characterized by negative phenotypic changes such as the ectopic deposition of lipid in times of energy excess. In previous studies, it was demonstrated that the botanical extracts generated from *Artemisia scoparia* (SCO) enhanced adipocyte differentiation and lipid accumulation, having positive metabolic consequences both in vitro and in vivo. In this study, we investigate the ability of SCO, and of three crude fractions of SCO, to modulate another important function of adipocytes: lipolysis. Lipolysis mobilizes adipocyte fat stores to maintain energy homeostasis. Insulin resistance is associated with increased rates of basal lipolysis and higher circulating fatty acids, compounding metabolic disruption.
Methods: Differentiated 3T3-L1 adipocytes were treated with an extract of SCO daily for 72 hours. To model the elevated lipolysis of metabolic syndrome, cells were treated with tumor necrosis factor alpha (TNFα) at 48 hours with a bolus at 2 hours before harvest. Lipolysis was measured utilizing an assay of extracellular glycerol, with and without SCO in the incubation media. In an independent experiment, adipocytes were treated with three crude fractions of the SCO extract to observe the relative impact on lipolysis using the same methods previously described.
Results: Basal lipolysis was unaffected or slightly elevated with SCO treatment. However, in the condition of elevated lipolysis that was achieved by stimulation with TNFα treatment, we observed a significant reduction with SCO treatment. The fractionation experiment indicated that one fraction had negligible effects on the lipolytic activity of treated cells. A second fraction appeared to recapitulate the effect of the parent extract, while the final fraction was toxic to the adipocytes at the experimental dose.
Conclusion: SCO decreases lipolysis after treatment with TNFα. The botanical has potential to combat unfavorable metabolic conditions associated with elevated basal lipolysis.

Background:

Metabolic syndrome, associated with epidemics of obesity and insulin resistance, is a burgeoning global health challenge [1, 2]. A growing population of individuals with non-communicable diseases, including metabolic syndrome and Type 2 Diabetes Mellitus, has created a new public health crisis. Diagnosis of metabolic syndrome is based on identification of at least three of the traditional risk factors of cardiovascular disease: visceral adiposity, hypertriglyceridemia, high fasting plasma glucose, impaired glucose tolerance, hypertension, and low HDL cholesterol [3]. A national survey identified Louisiana at the center of this problem, with more than 35% the adult population self-reporting as obese in 2016 [4, 5]. These patients require long term and careful medical attention, putting additional strain on the state's already resource-poor health care delivery system.

Metabolic syndrome results in negative phenotypic changes, visible at the cellular level. Adipocytes are recognized as cells active in the regulation of metabolism, with roles in lipid storage, insulin sensitivity and endocrine function. Adipocytes produce the hormone adiponectin, which acts to stimulate adipocyte growth and maturation as well as improve the insulin sensitivity of the liver, skeletal muscle, and other tissues [6]. Disruption of regular adipocyte growth and action is a primary contributor to metabolic syndrome [7, 8].

In times of energy excess, the expansion of adipose tissue provides safe storage for lipids. Dietary fat is stored in the lipid droplets of adipocytes as relatively inert triacylglycerol (TAG). TAG stores are essential in the fasted state, because they can be catabolized to maintain blood glucose and energy homeostasis. Lipolysis converts TAG to

more active substrates: glycerol and non-esterified free fatty acids (NEFAs). Glycerol and NEFAs are released from adipocytes to meet energy needs. In the fed state, with excess energy available, healthy individuals experience lower levels of lipolysis because there is no need to access the TAG stores of adipocytes. However, insulin resistance, a result of obesity and high fat diet, is associated with elevated basal lipolysis and higher circulating fatty acids [9]. Even in the fed state, insulin resistant individuals fail to downregulate lipolysis, compounding the effect of a high fat diet on excess fat in the blood. The ectopic deposition of lipid in peripheral tissues, a byproduct of increased levels of lipolysis, inhibits organ function and further disrupts metabolism [6].

Pharmaceutical intervention is an important tool for combatting metabolic syndrome. Botanicals are an important and well-established source for the derivation of medication and dietary supplements. In fact, approximately one-third to one-half of modern medications are derived from botanicals [10]. A particularly relevant example is Metformin, the first line of defense and a leading pharmaceutical treatment for Type 2 Diabetes, which is derived from a compound present in French Lilac. We have investigated several plant extracts for their potential to target the negative pathological consequences of metabolic syndrome. Previous studies have shown that an extract of the botanical *Artemisia scoparia* (SCO) acts to promote fat cell differentiation, improve insulin sensitivity, and lower fasting blood glucose in an obese mouse model [11, 12].

In order to better understand the mechanisms of these positive metabolic effects, this in-vitro study examines the ability of SCO to modulate lipolysis in cultured mouse adipocytes. A whole-plant extract, which contains at least hundreds of different compounds, was used to assess the regulation of lipolysis in fat cells. In addition, three

crude fractions of the extract were tested to make progress towards identifying the metabolically active component of SCO.

Materials and Methods:

SCO Extract: A partner of the Stephens Lab at Rutgers prepared an ethanolic extract of the entire *Artemisia scoparia* plant, which was solubilized in dimethyl sulfoxide (DMSO). Three crude fractions of SCO were prepared in a similar manner.

Cell Culture and Treatment: 3T3-L1 preadipocytes were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). They were differentiated according to protocol, and media was changed every 48-72 hours. Fully differentiated, mature adipocytes were used for the lipolysis experiment. For the length of the treatment, the cells were maintained in DMEM with 10% FBS, changed daily. Adipocytes were treated daily for 72 hours with SCO extract at 50 μg/ml, SCO crude fractions at 5 μg/ml and 20 μg/ml, or DMSO vehicle. At 48 hours, the cells were also treated with 0.75 nM TNFα or vehicle (BSA in PBS). For the last 2 hours, the cells received an additional bolus of TNFα or vehicle at the same concentration, added to the existing media.

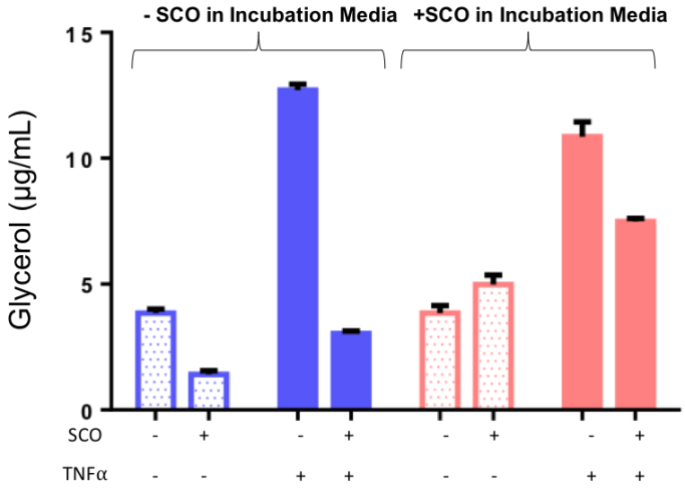
Lipolysis Assay: After the additional 2-hour exposure to TNFα, the treatment media was replaced with incubation media. Since the SCO extract contains many different compounds, which could have biochemical activity that interferes with the enzymatic assay, SCO treatment was continued in the incubation media as an additional controlled condition. The incubation media, 2% BSA + 0.1% glucose in DMEM, was collected after two hours and used in the assay. To get sufficient signal within the standard range, the assay was performed with 50μl sample/well. The Sigma Free Glycerol Assay was used to measure

extracellular glycerol. Lipolysis releases both glycerol and non-esterified free fatty acids, so extracellular glycerol was used to approximate lipolysis. The assay relies on a series of enzymatic reactions to produce hydrogen peroxide, culminating in a proportional colorimetric product.

Results:

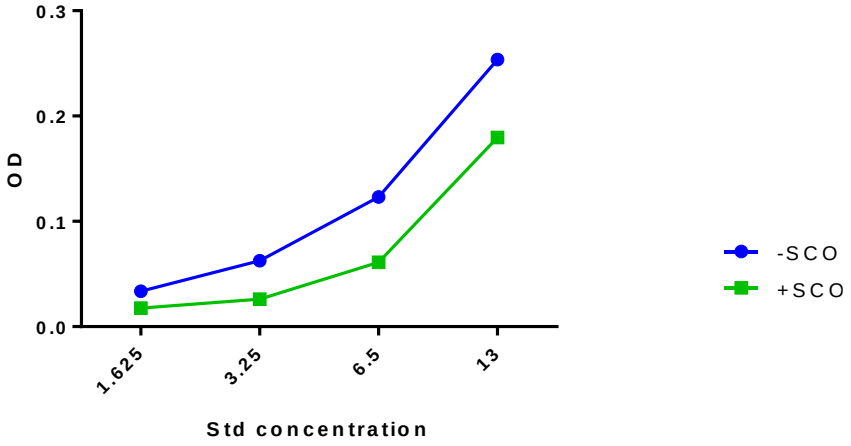
In the initial assay, there was an expected, observable induction of lipolysis in the TNFα-stimulated condition. As shown in Figure1, the addition of SCO in the incubation media markedly reduced lipolysis at both basal and TNFα-stimulated conditions. However, when SCO was excluded from the incubation media, the robust effect of SCO on basal lipolysis was lost. In fact, without SCO in the incubation media, SCO pretreatment resulted in a slight increase in lipolysis at basal conditions. Although the effect was not as robust without SCO in the incubation media, lipolysis was significantly reduced after the 72-hour pretreatment at the TNFα-induced condition.

Figure 1. SCO Reduces TNFα Induced Lipolysis . 3T3-L1 adipocytes were treated for 72 hours with SCO extract at 50 μg/ml; TNFα was added after 48 hours at 0.75nM, with an additional 2-hour bolus before media was replaced for incubation (with or without SCO) and harvested to assay extracellular glycerol.



As a whole-plant extract, SCO contains many unique, potentially active compounds. Since the changes in extracellular glycerol after SCO treatment were significantly different based on the presence of SCO in the final incubation media, further investigation was needed to elucidate any effects of SCO extract on the enzymatic reactions of the lipolysis assay. A series of standard curves, made up of known concentrations of glycerol, were evaluated via assay protocol with and without the addition of SCO, shown in Figure 2. The curve which included SCO treatment showed a reduction in glycerol concentration based on the assay's final colorimetric product, reflected by the green line in Figure 2. The comparative reduction in measured glycerol from the known values, accurately measured by the assay without SCO treatment and shown in blue, occurred at every concentration.

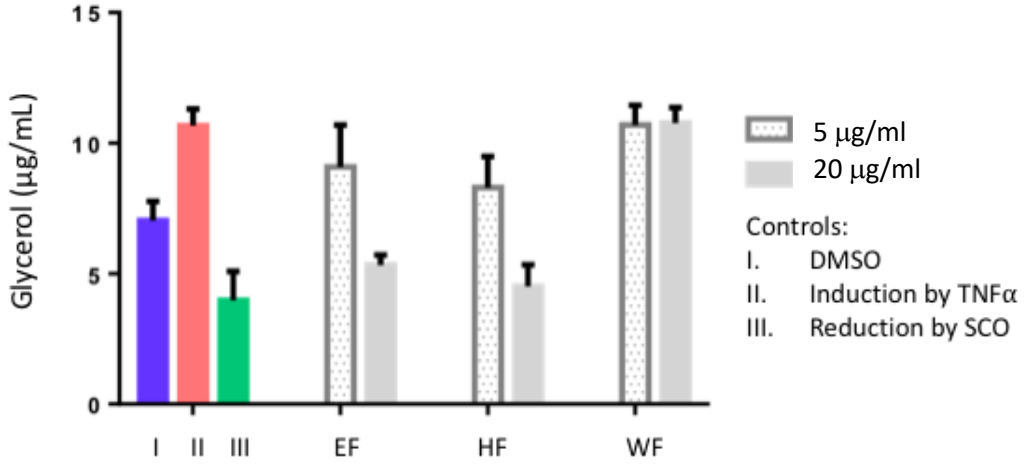
Figure 2. SCO Interference Results in Artificially Low Measurements of Glycerol Concentrations. Known concentrations of glycerol were measured, with and without the presence of SCO, by the assay's final colorimetric product.



To begin identifying the active component(s) of SCO, the three crude fractions were tested using the same protocol as was performed with the parent extract. The TNFα-stimulated conditions, shown to be relevant by experimental analysis of the parent extract, are displayed in Figure 3.

As shown in Figure 1, TNFα induces lipolysis well above basal levels, and the presence of the SCO parent significantly reduces TNFα-induced lipolysis in adipocytes. The three crude fractions of SCO, named Fraction E (EF), Fraction H (HF), and Fraction W (WF), had differing effects on lipolysis, displayed by Figure 3. Fraction E showed a dose-dependent reduction in lipolysis, mirroring the effect of the parent extract at 20 μg/ml. Cells treated with Fraction H also showed a decrease in extracellular glycerol. However, during the 72-hour pretreatment, adipocytes treated with Fraction H appeared stressed, with notable cell death (data not shown). The third extract, WF, shows little to no effect on TNFα-induced lipolysis, at 5 μg/ml or 20 μg/ml. Of the three crude fractions, only fraction E was able to recapitulate the effects of the parent extract without cytotoxicity at the experimental concentrations.

Figure 3. Treatment with Three Crude Fractions of SCO alters extracellular glycerol levels. 3T3-L1 adipocytes were treated for 72 hours with fraction extracts; TNFα was added after 48 hours at a concentration of 0.75nM, with an additional 2-hour bolus before media was replaced for incubation and harvested to assay extracellular glycerol.



Discussion:

Adipocytes play an important role in maintaining metabolic health. In models of obesity and insulin resistance, adipocytes fail to function normally, leading to elevated glycerol and circulating free fatty acids, even at basal conditions. Although obesity is characterized by excess adipose tissue, the metabolically relevant actions of adipocytes, lipid storage, insulin sensitivity, and endocrine function, are impaired. In addition, the tissue expands abnormally, often with extreme hypertrophy or hyperplasia. This dysregulation at the cellular level provides a potential target for therapeutic intervention via adipocyte modulation.

The magnitude and morbidity of the current metabolic crisis necessitate new strategies for intervention. Botanicals have a precedent in traditional and complementary medicine that is well-documented. However, there is a need to investigate the biological mechanisms that mediate the metabolic activity of botanicals. Our lab has demonstrated that one such botanical, *Artemisia scoparia* (SCO), has relevance for the promotion of positive effects on adipocyte functions [11, 12]. In the present in-vitro study, we examined the effects of SCO on lipolysis, an adipocyte function that is critical in providing energy.

Chronic exposure to the SCO botanical extract over the course of three days had a significant impact on TNFα-induced lipolysis in mature adipocytes. In the initial screening, it appeared that SCO was able to lower the rates of lipolysis across all metabolic conditions. However, when SCO was removed from the final, 2-hour incubation step, the large reductions in lipolysis were no longer observed. At basal conditions, SCO treatment had little effect on lipolysis, causing a slight increase. At TNFα-stimulated conditions of elevated

lipolysis, SCO retained some of the previously observed effects, although the reduction in lipolysis was lessened shown in Figure 1.

Further investigation suggests that SCO, which is a complex whole-plant extract containing many chemical compounds, interferes directly with the assay used to measure extra-cellular glycerol. As demonstrated in Figure 2, the addition of SCO extract to known quantities of glycerol, without exposure to the biologically active cellular machinery of adipocytes, showed an artificial reduction in the measurements of glycerol. The lipolysis assay depends on a series of enzymatic steps. First, extracellular glycerol is phosphorylated. This compound is converted to hydrogen peroxide, which is then directly proportional to the final colorimetric product.

Since the effects of SCO occur over the course of 72 hours, it is unlikely that the final 2-hour exposure to SCO in the incubation step has any substantial biological relevance. Instead, there is evidence to support that the extract acts as a reducing agent to interfere with the production of H2O2 in the enzymatic assay, thereby impairing the accurate measurement of extracellular glycerol. The extreme reduction of lipolysis across all conditions, observed in the initial screening, is now thought to be an artifact of chemical interference with the glycerol assay. However, an observable reduction in lipolysis still occurred without SCO in the incubation media when adipocytes were stimulated with TNFα. Pre-treamtent with SCO does not appear to interfere with the assay's ability to measure extracellular glycerol, indicating a legitimate decrease in the lipolysis of adipocytes in-vitro.

The TNFα-stimulated condition has significant metabolic relevance in the context of elevated basal lipolysis that is observed in obesity and insulin resisitance in both mouse

and man [13] The reduction in lipolysis that is observed in this study indicates that *Artemisia scoparia* has biological potential to ammeliorate some of the negative physiological effects associated with Metabolic Syndrome. To work towards identifying of the active component of SCO present in the whole-plant extract, the 72-hour pretreament protocol was repeated with three crude fractions of SCO (EF, HF, WF) alongside the parent extract. Adipocytes were stimulated with TNFα and lipolysis was analyzed via colrimetric assay of extracellular glycerol without SCO in the incubation media.

As previously demonstrated, TNFα induced lipolysis was reduced in the presence of the SCO parent extract. The three fractions were administered at two lower concentrations to approximate the quantity of biologically active material found in the parent extract. While fractions EF and HF had observable effects on extra-cellular glycerol, WF showed no significant change from the TNFα-stimulated control (Figure 3). In addition, a dose-dependent effect of EF and HF was demonstrated by the assay. The concentration of extracellular glycerol was inversely correlated with the concentration of fraction treatments. During pretreatment, cells treated with HF appeared stressed, with observable cell death over the course of 72 hours (data not shown). The decrease in extracellular glycerol was possibly due to less living cells rather than less lipolysis. EF appears to be the only fraction able to recapitulate the effects of the parent extract without significant cytotoxicity at tested concentrations. Further study is required to test the fraction extracts at lower concentrations to determine the relevant alterations in lipolysis. Further fractionation of the EF extract will hopefully lead to the identification of one or more compounds that mediate the ability of SCO to reduce TNFα-induced lipolysis in adipocytes.

Conclusion:

This in-vitro study supports previous findings that *Artemisia scoparia* has the ability to modulate adipocyte function in a metabolically beneficial manner. The reduction of extracellular glycerol after TNFα-stimulation provides evidence that the botanical may be able to mediate the negative effects of insulin resistance on basal lipolysis. Further study is merited to identify biologically active components of the SCO botanical as well as investigate the cellular mechanisms by which the observed changes in lipolysis occur. In light of the growing global health crisis associated with obesity and Type 2 Diabetes Mellitus, *Artemisia scoparia* is a promising target for pharmaceutical development with potential to combat the physiological dysfunction associated with metabolic syndrome.

References:

1. Unger RH, Clark GO, Scherer PE, Orci L. Lipid homeostasis, lipotoxicity and the metabolic syndrome. *Biochim Biophys Acta.* 2010;1801(3):209–214.
2. Ford, E. S.; Giles, W. H.; Dietz, W. H. Prevalence of the metabolic syndrome among US adults: findings from the third National Health and Nutrition Examination Survey. JAMA 2002, 287, 356–9, doi:10.1001/jama.287.3.356.
3. Roberts C.K., Hevener A.L., Barnard R.J. Metabolic syndrome and insulin resistance: Underlying causes and modification by exercise training. Compr. Physiol. 2013;3 doi: 10.1002/cphy.c110062.
4. Johnson NB, Hayes LD, Brown K, Hoo EC, Ethier KA. CDC National Health Report: leading causes of morbidity and mortality and associated behavioral risk and protective factors—United States, 2005–2013. *MMWR Surveill Summ*. 2014;63 Suppl 4:3–27.
5. Ward ZJ, Long MW, Resch SC, et al. Redrawing the US obesity landscape: bias-corrected estimates of state-specific adult obesity prevalence. PLoS One. 2016;11(3):e0150735.
6. Attie AD, Scherer PE. Adipocyte metabolism and obesity. *J Lipid Res.* 2009;50 suppl:S395–S399.
7. Kim JY, van de Wall E, Laplante M, Azzara A, Trujillo ME, et al. (2007) Obesity-associated improvements in metabolic profile through expansion of adipose tissue. J Clin Invest 117: 2621–2637 doi: 10.1172/JCI31021.
8. Danforth E Jr (2000) Failure of adipocyte differentiation causes type II diabetes mellitus? Nat Genet 26: 13. doi: 10.1038/79111.
9. Xu H, et al. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest.* 2003;112(12):1821–1830.
10. Veeresham P, (Dr) C. Natural products derived from plants as a source of drugs. J. Adv. Pharm. Technol. Res. 2012;3:200. doi: 10.4103/2231-4040.104709.
11. Richard AJ, Fuller S, Fedorcenco V, Beyl R, Burris TP, Mynatt R, et al. (2014) *Artemisia scoparia* Enhances Adipocyte Development and Endocrine Function *In Vitro* and Enhances Insulin Action *In Vivo*. PLoS ONE 9(6): e98897. doi:10.1371/journal.pone.0098897.
12. Richard A. J., Burris T. P., Sanchez-Infantes D., Wang Y., Ribnicky D. M., Stephens J. M. Artemisia extracts activate PPAR*γ*, promote adipogenesis, and enhance insulin sensitivity in adipose tissue of obese mice. *Nutrition*. 2014;30(7-8):S31–S36. doi: 10.1016/j.nut.2014.02.013.
13. Nguyen MT, et al. JNK and tumor necrosis factor-alpha mediate free fatty acid-induced insulin resistance in 3T3-L1 adipocytes. *J Biol Chem.* 2005;280(42):35361–35371.