The Role of Herbal Extracts on Fat Metabolism

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CHAPTER 1 LITRATURE REVIEW

Triacylglycerol (TAG)

I Introduction

TAG, one of the major neutral lipids, is composed of three fatty acids (FAs) esterified to a glycerol backbone. The three FAs influence the chemical and physical characteristics of TAG by the length of carbon chains and degree of desaturation. The esterification happens between three hydroxyl groups (-OH) on the glycerol molecule and carboxyl groups (-COOH) on each of the three FA molecules. The most important function of TAGs is to efficiently store energy in the form of FAs, thereby preventing the potential toxicity of excessive free fatty acids (FFAs). Assembled and secreted in mammal intestines and livers, TAGs are also important for transporting FAs to different tissues throughout the body (Yen, Stone, Koliwad, Harris, & Farese, 2008). In addition to their important role in energy storage, TAGs also have other roles in the body. Being one of the predominant components of stratum corneum lipids, TAGs is very important for the water barrier on the surface of skins (Downing et al., 1987). TAGs also provide a protective padding and an insulation preventing heat loss for organisms (Budd, Brotherhood, Hendrie, & Jeffery, 1991). However, abnormal TAG accumulation can result in adverse health outcomes. Excessive TAG accumulation in adipose tissue defines obesity (Allison & Saunders, 2000). In nonadipose such as livers, skeletal muscle and hearts, excessive deposition of TAGs results in certain organ dysfunctions like insulin resistance, nonalcoholic steatohepatitis and cardiomyopathy (Unger, 2002) (Friedman, 2002). On the other hand, lack of fat accumulation in adipose tissue by genetic or acquired means is called lipodystrophy, which is also related to insulin resistance and

other health disorders (Garg, 2006).

II The synthesis of TAG

There are two major pathways for the synthesis of TAG as shown in Figure 1; the glycerol phosphate pathway and the monoacylglycerol (MAG) pathway (Yen et al., 2008). The glycerol phosphate pathway, which is also the de novo route for TAG biosynthesis, happens in most cell types while the MAG pathway has a primary role in enterocytes, although it may also contribute to TAG metabolism in adipocytes and hepatocytes where it may be involved in TAG reesterification (Xia, Mostafa, Bhat, Florant, & Coleman, 1993). Both pathways need fatty acyl-CoAs as acyl donors, which are synthesized by acyl-CoA synthetases inside of cells (Coleman, Lewin, Van Horn, & Gonzalez-Baro, 2002).

The first three steps in the glycerol phosphate pathway are catalyzed by glycerol-phosphate acyltransferase (GPAT) to form lysophosphatidate (LPA), acylglycerol-phosphate acyltransferase (AGPAT) to produce phosphatidate (PA) and phosphatidic acid phosphohydrolase (PAP) to form DAG (Bell & Coleman, 1980). In the MAG pathway, there is only one enzyme needed for synthesis of DAG from MAG, which is monoacylglycerol acyltransferase (MGAT) (Lehner & Kuksis, 1996) (Coleman & Lee, 2004). Although the enzymes involved in these pathways differ, both pathways share the terminal reaction - joining a fatty acyl-CoA and DAG together to form TAG, which is the committed step in TAG biosynthesis that is catalyzed by diacylglycerol acyltransferases

(DGATs) (Cases et al., 1998).

The activity of GPAT is the lowest among all the enzymes involved in the glycerol phosphate pathway suggesting this first step be rate-limiting (Bell & Coleman, 1980). GPAT exists in microsomes and mitochondria. Microsomal GPAT in liver contributes 50% to 80% of total GPAT activity and in most other tissue it contributes up to 90% of total activity (Bell & Coleman, 1980). Microsomal isoforms play a very important role fat synthesis. When 3T3-L1 adipocytes differentiate and TAG synthesis increases in neonatal livers, the specific activity of microsomal GPAT increases 70-fold and 74-fold respectively while mitochondrial GPAT (mtGPAT) has only 10-fold and 5-fold (Coleman & Bell, 1980) (Coleman & Haynes, 1983). MtGPAT is sensitive to changes in diet and hormone levels which is consistent with its role in TAG synthesis (Dircks & Sul, 1997) (Coleman, Lewin, & Muoio, 2000). Under fasting status hepatic mtGPAT levels and activity decreased, both of which increased after a high-carbohydrate diet (Lewin, Granger, Kim, & Coleman, 2001). MtGPAT is also downregulated in streptozotocin-diabetic mice and insulin brings the expression level of mtGPAT up to about 20-fold (Shin, Paulauskis, Moustaid, & Sul, 1991).

AGPAT is the enzyme that catalyzes the reaction of phosphatidate synthesis. AGPAT 1 and AGPAT 2 have been the most studied of the AGPAT 1-6 family (Coleman & Lee, 2004). In humans, AGPAT 1 exists mainly in liver, heart, immune tissues and epithelium while AGPAT 2 is highly expressed in human liver and heart (Eberhardt, Gray, & Tjoelker, 1997) (West et al., 1997). PAP, also known as lipin, catalyzes next step for the

synthesis of DAG from phosphatidate. There are three isoforms of PAP discovered in mammals (Reue & Zhang, 2008). PAP 1 is the only type involved in TAG synthesis among different types of PAP (Jamal, Martin, Gomez-Munoz, & Brindley, 1991).

MGAT is the only enzyme in the MAG pathway. The activity of MGAT is much higher in small intestine than in liver, adipose tissue, kidney, skeletal muscle and heart (Yen, Stone, Cases, Zhou, & Farese, 2002). Three different types of MGAT have been studied and all of them are found in the endoplasmic reticulum (Cao, Lockwood, Burn, & Shi, 2003) (Cheng et al., 2003). In mice, MGAT 2 and 3 are highly expressed in the small intestine but MGAT 1 is not, indicating that MGAT 2 and 3 are the primary types playing an important role in TAG synthesis (Coleman & Lee, 2004).

DGAT 1 and DGAT 2 catalyze the formation of TAG from DAG. In humans, DGAT 1 is highly expressed in adipose tissue and the small intestine, and DGAT 2 is mainly in liver, adipose tissue, mammary gland and testis (Cases et al., 1998) (Cases et al., 2001). When the expression level of DGAT 1 increases in mice, the size of fat cells becomes larger (H. C. Chen, Stone, Zhou, Buhman, & Farese, 2002). With an acute fed of high-fat diet, DGAT 1 deficient mice did not have increased TAG levels in plasma comparing to wild type mice, suggesting that DGAT 1 is crucial for the synthesis of TAG-rich chylomicra (Buhman et al., 2002). But with a chow diet, DGAT 1 deficient mice still have normal TAG levels in circulation and TAG storage, showing the importance of DGAT 2. DGAT2 deficiency in mice caused significant fat reduce and death, which cannot be

compensated by DGAT1 (Stone et al., 2004). Thus, both DGATs appear important for TAG synthesis although much remains unknown about specific functions of each isoform.

III The hydrolysis of TAG

The hydrolysis of TAG (Figure 2), also known as lipolysis, is a major mechanism to supply fatty acids for intracellular use or, in the case of adipose tissue, to other organs (Duncan, Ahmadian, Jaworski, Sarkadi-Nagy, & Sul, 2007a). Lipolysis is an ordered and well-regulated process, of which each step is catalyzed by a different enzyme. There are three major enzymes in this pathway: adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and MAG lipase. ATGL facilitates the breakdown of one fatty acid from TAG, producing DAG (Villena, Roy, Sarkadi-Nagy, Kim, & Sul, 2004). The second fatty acid is hydrolyzed from DAG by HSL. MAG lipase then breaks down MAG to the final fatty acid and a glycerol molecule (Ahmadian, Duncan, Jaworski, Sarkadi-Nagy, & Sul, 2007).

ATGL (desnutrin) was indentified and characterized in 2004 (Villena et al., 2004). ATGL is mainly expressed in adipose tissue, but it is also found in other tissues such as heart and skeletal muscles (Duncan, Ahmadian, Jaworski, Sarkadi-Nagy, & Sul, 2007b). In vitro studies on COS-7 cells and 293HEK cells showed that ATGL overexpression decreased intracellular TAG storage and increased the levels of free fatty acids (FFAs) in the medium (Villena et al., 2004) (Lake et al., 2005). ATGL knockdown in 3T3-L1 adipocytes decreases FFAs release to the medium (Zimmermann et al., 2003). Mice with ATGL deficiency gain weight and have ectopic fat accumulation in tissues such as heart

(Haemmerle et al., 2006). Moreover, ATGL deficient mice use carbohydrate over fat as the major fuel for energy (Haemmerle et al., 2006). Fasting status induces ATGL expression and refeeding suppresses it, suggesting that ATGL is regulated by nutritional status (Duncan, Ahmadian, Jaworski, Sarkadi-Nagy, & Sul, 2007a).

HSL is a lipase with a broad range of substrates such as cholesteryl esters, retinyl esters, DAG and TAG, among which the relative maximal hydrolysis rate for DAG is the highest (Holm, 2003). The role of HSL in fat hydrolysis has been illustrated in HSL knockout animals. The size of adipocytes in HSL knockout mice became larger and total TAG lipase activities in adipose tissue decreased (Osuga et al., 2000). Additionally, the levels of FFAs and TAG in circulation were reduced in HSL deficient mice (Haemmerle, Zimmermann, Strauss et al., 2002). HSL deficiency causes DAG accumulation in tissues including adipose tissue, skeletal muscle and heart (Haemmerle, Zimmermann, Hayn et al., 2002) suggesting that HSL plays an important role in TAG hydrolysis and FFAs mobilization, but it is not necessary for the initial step of TAG hydrolysis.

MAG lipase catalyzes in the last step of lipolysis. It only catalyzes hydrolysis of MAG, with no specific activity against DAG and TAG (Karlsson, Contreras, Hellman, Tornqvist, & Holm, 1997). Yet, despite is important role in this pathway, this enzyme has not been extensively characterized.

Some lipid droplet proteins also regulate lipolysis rates such as adipose fatty acid-binding protein (aFABP), caveolin and members of the PAT protein family including perilipin.

Perilipin is one of the major lipid droplet proteins in adipocytes which has two isoforms, perilipin A and B (Greenberg et al., 1993). The expression level of perilipin A is much higher that perilipin B. Mice missing perilipin are leaner and with smaller fat cells and are resistant to diet-induced obesity (Martinez-Botas et al., 2000) (Saha, Kojima, Martinez-Botas, Sunehag, & Chan, 2004). Ectopic expression of perilipin A in 3T3-L1 preadipocytes leads to more intracellular TAG accumulation and slower lipolysis than control cells (Brasaemle et al., 2000). The presence of perilipin is to restrict the access of TAG lipases to lipids and restrain basal lipolysis. However, perilipin null mice also exhibit significant attenuation of stimulated lipolysis, suggesting that perilipin is necessary to maintain a regular level of lipolysis (Tansey et al., 2001). Another important lipid droplet protein, comparative gene identification-58 (CGI-58), is an activator of ATGL (Lass et al., 2006). Mutations in CGI-58 lead to excessive TAG accumulation in several tissues in human (Lass et al., 2006). Overexpression of CGI-58 and ATGL at the same time reduces intracellular TAG accumulation significantly more comparing to overexpressing CGI-58 or ATGL alone in COS-7 cells (Lass et al., 2006). Normally CGI-58 locates on the surface of lipid droplets in adipocytes by interacting with perilipin and it only associates with approximately 25% of ATGL (Shen, Patel, Miyoshi, Greenberg, & Kraemer, 2009). Under lipolytic stimulated conditions, the interaction between CGI-58 and ATGL increases significantly. Thus more ATGL moves to lipid droplets from cytosol (Granneman et al., 2007).

Adipose tissue

I Introduction

Adipose tissue makes up approximately 15-20% of the body weight of people with normal weight. Adipose tissue is primarily composed of adipocytes although other cell types such as fibroblasts, macrophages, and endothelial cells are also present. Adipose is the major tissue for storage of energy reserves in the form of TAGs. Adipose tissue is divided into two types, white adipose tissue (WAT) and brown adipose tissue (BAT), between which adipocytes exhibit significant differences. The diameters of adipocytes in WAT are on average 60-100 µm while adipocytes in BAT are about 30-40 µm (Fonseca-Alaniz, Takada, Alonso-Vale, & Lima, 2007). Adipocytes in WAT have unilocular lipid droplets whereas BAT has numerous smaller lipid droplets. There is a significant amount of blood vessels and mitochondria in BAT giving BAT a brown color and a function for non-shivering thermogenesis which is critical for hibernating and neonatal animals and, as recently discovered, may play a role in energetics in adult humans as well (Cannon & Nedergaard, 2004) (Cypess et al., 2009). In humans, adipose tissue is mainly located under the skin, around internal organs and in the yellow bone marrow (Gurevitch, Slavin, & Feldman, 2007). Based on these different locations, adipose tissue can be further classified as subcutaneous and visceral adipose tissue (Shoelson, Herrero, & Naaz, 2007).

II As a TAG storage organ

The major sources of TAG for adipose tissue are diet and *de novo* lipogenesis. Dietary saturated and unsaturated fatty acids can be used to form TAG directly or taken up from

very low-density lipoprotein (VLDL) and chylomicrons after digestion. *De novo* lipogenesis refers to TAG synthesis in the body utilizing other sources such as carbohydrate under a positive energy balance (Jeffcoat, 2007).

A primary function of adipose tissue is to store excess calories in the form of TAG. Given that TAG provides 9 kcal/g while carbohydrate and protein only contain 4 kcal/g, TAG storage in adipose tissue allows the body to store additional calories very efficiently. Another important benefit of storing TAG in adipose tissue is to lower serum free fatty acids (FFAs). An elevated level of free fatty acids (FFAs) in circulation has been confirmed as one of the major causes for insulin resistance (Boden, 1997) (Boden & Shulman, 2002) (McGarry & Dobbins, 1999). Esterification of FFAs to TAG and transferring to adipose tissue efficiently prevents the toxic burden of ectopic lipid overload in nonadipose tissue (Unger, Clark, Scherer, & Orci, 2010).

However, excessive TAG synthesis resulting in increased TAG storage in adipose tissue will lead to another morbid state, obesity. The number of adipocytes is established during childhood for human beings (Knittle, Timmers, Ginsberg-Fellner, Brown, & Katz, 1979). It does not change during the development of obesity in adults (Willard, 1991), except in morbid obesity (Garaulet, Hernandez-Morante, Lujan, Tebar, & Zamora, 2006). Thus, a proper amount of TAG storage in adipose tissue is necessary to maintain normal metabolic regulation.

III Adipose as an endocrine organ

Adipose tissue not only stores energy, but is also a major endocrine organ. Adipokines are proteins secreted by adipocytes that act as signaling molecules to other tissues influencing a multitude of pathways including energy metabolism (Muoio & Newgard, 2005). One of the most studied adipokines is leptin, which was first identified as the gene responsible for the obese phenotype of ob/ob mice (Zhang et al., 1994). Secreted mainly by adipose tissue (Lee et al., 2006), leptin is critical in regulating body weight by modulating food intake and increasing energy expenditure. Administration of leptin via intraperitoneal injections or subcutaneous infusion decreases body weight in both lean and obese mice (Halaas et al., 1997) (Halaas et al., 1995) (Pelleymounter et al., 1995). Leptin is secreted in proportion to adipose mass; thus, it is typically high in obese individuals and low in subjects with lower levels of adiposity (Kennedy et al., 1997). Acute changes in energy intake also regulate leptin secretion. For example, short term food restriction reduces leptin secretion regardless of the level of adiposity (Tapsell et al., 2010). Leptin acts through binding one of several leptin receptors expressed throughout the body to involve in food intake and energy expenditure

Adiponectin is another well-studied polypeptide hormone, which is predominantly secreted by adipose tissue. Different from leptin, levels of adiponectin in plasma are inversely correlated to the amount of body fat (Ukkola & Santaniemi, 2002). The adiponectin levels in circulation are normally very high in healthy people, which ranges between 5–30 mg/L (Garaulet, Hernandez-Morante, de Heredia, & Tebar, 2007). It was thought that adipocytes were the only type of cells which could synthesize adiponectin

until further research revealed that adiponectin can also be secreted at lower levels by other cells such as hepatocytes, skeletal muscle cells and colonic mucosa cells (Nishida, Funahashi, & Shimomura, 2007) (Matsunaga et al., 2008). Based on configurations and molecular weights, there are six isoforms of adiponectin (Beltowski, Jamroz-Wisniewska, & Widomska, 2008) (Giannessi, Maltinti, & Del Ry, 2007). Sufficient research and clinical studies have indicated that adiponectin is a protective factor against diseases, such as insulin resistance, diabetes and atherosclerosis(Beltowski et al., 2008) (Karbowska & Kochan, 2006) (Kelesidis, Kelesidis, & Mantzoros, 2006) (Nishida et al., 2007).

IV Adipose tissue and Type II diabetes (T2D)

T2D, one of the obesity-related metabolic disorders, occurs when body does not response to insulin causing high blood glucose that ultimately leads to the pancreatic failure. Based on data from the American Diabetes Association, 23.6 million people in the United States had diabetes in 2007, which is 7.8% of the population. From 1997 to 2003, the incidence of diagnosed diabetes was raised to 6.9‰ from 4.9‰ in the US (Geiss et al., 2006). Studies consistently showed that diabetes is more prevalent among obese people (Millar & Young, 2003). In 2003 about 30% of diabetic patients were overweight and 59% were obese (Geiss et al., 2006).

Besides total fat mass, the distribution of body fat appears to be important in the development of insulin resistance and T2D (Nam et al., 2010). Fat deposited in insulin-sensitive tissue such as muscle, liver and pancreas can decrease insulin sensitivity leading

to T2D (Nam et al., 2010). Based on this point, adipose tissue can also be a "protective" factor against T2D. Fat located outside of adipose tissue is more likely to cause insulin resistance than TAG stored within adipose tissue. Mice lacking fat tissue showed severe insulin resistance and increased intracellular lipid accumulation in liver and skeletal muscle (J. K. Kim, Gavrilova, Chen, Reitman, & Shulman, 2000). Additionally, adipose tissue secretes hormones such as adiponectin that can increase effectiveness of insulin. Animal studies showed that adiponectin stimulates the expression of PPARγ target genes and reduces adipose tissue macrophage infiltration (J. Y. Kim et al., 2007). Some people with high levels of adiponectin can be obese but still be protected against T2D (Vasseur, Meyre, & Froguel, 2006). From this aspect, certain amount of fat mass can be a protective factor against T2D.

Inflammation and metabolic disorders

Obesity and insulin resistance are typically related to inflammation. High-fat fed mice that develop obesity have increased expression of genes related to inflammation in white adipose tissue (Xu et al., 2003). The increase of TAG and inflammatory response occurs simultaneously, but prior to the development of hyperinsulinemia. Therefore, it is reasonable to think that excess fat accumulation can trigger insulin resistance.

In the obese state, excessive adiposity often accumulates not only in adipose tissue, but also in skeleton muscles and liver, which results in insulin resistance and increasing levels of inflammatory molecules in blood (Stienstra, Duval, Muller, & Kersten, 2007).

As mentioned before, adipose tissue itself can secrete a variety of adipokines that act as links between obesity and related metabolic disorders like insulin resistance, Type II diabetes and cardiovascular diseases. The first study showing the direct relationship between obesity and inflammation was by Hotamisligil et al, who found that the expression of TNF-α increases in obese and diabetic rodent models and neutralization of TNF-α can improve insulin sensitivity in peripheral tissues (Hotamisligil, Shargill, & Spiegelman, 1993). Since these initial studies, numerous animal studies have confirmed that inflammation plays a key role in the development of obesity-associated comorbidities.

Besides adipocytes and connective tissue matrix, adipose tissue also contains a variety of immune cells such as macrophages (Hotamisligil et al., 1993). The numbers of macrophages in adipose tissue are correlated to fat mass and size of adipocytes in both human beings and mice (Weisberg et al., 2003). Obesity commonly causes macrophage infiltration, which is triggered in part by monocyte chemoattractant protein-1 (MCP-1) produced in adipose tissue (Kanda et al., 2006). Macrophages also produce more proinflammatory factors such as MCP-1 and interleukin-6 (IL-6) (Xu et al., 2003). The infiltration of macrophages can stimulate insulin resistance as discussed before. Numerous studies show that levels of cytokines especially IL-1, IL-6 and TNF-α are upregulated among patients with Type II diabetes or animal models of insulin resistance (Pickup & Crook, 1998).

Major inflammatory factors

I Nuclear factor-κB (NF-κB)

NF-κB is a transcription factor that plays an important role in regulating inflammatory pathways. NF-κB responds to various stimuli such as cytokines and oxidized LDL (low density lipoprotein) (Robbesyn, Salvayre, & Negre-Salvayre, 2004). The expression of NF-κB protein can be stimulated by lipopolysaccharide (LPS) and cytokines such as tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β). More than a decade ago, tumor necrosis factor (TNF)-α was found to be highly expressed in adipose tissues of obese rodents (Hotamisligil et al., 1993). Uysal KT et al. investigated the role of TNF-α in obesity and diabetes. Using diet-induce obese mice, they found that mice lacking TNF-α or TNF-α receptors were protected from diet-induced insulin resistance (Uysal, Wiesbrock, Marino, & Hotamisligil, 1997). These studies show that TNF-α is one of the molecular links between inflammation and metabolic disorders.

NF-κB exists in an inactive form bound to IκB, a cytoplasmic inhibitor of NF-κB (Baeuerle & Baltimore, 1988). Binding to NF-κB, IκB is able to mask signals for nuclear translocation of NF-κB, keeping it in the cytoplasm as a kinase complex (IKK) under normal conditions (Verma, Stevenson, Schwarz, Van Antwerp, & Miyamoto, 1995). Activated by some signals such as tumor necrosis factor alpha (TNF-a) and lipopolysaccharide (LPS), the IκB is phosphorylated leading to its degradation (Van Antwerp & Verma, 1996). Subsequently, NF-κB is released and translocates to the nucleus activating expression of many important inflammatory molecules such as IL-6,

MCP-1 and TNF-α (Leeman & Gilmore, 2008).

Given the link between inflammation and metabolic diseases, it is not surprising that several studies show that anti-inflammatory drugs improve insulin resistance. High-dose sodium salicylate, which has anti-inflammatory functions, improves insulin resistance and glycosuria in humans (Williamson, 1901). It has also been found that high doses of aspirin improved hyperglycemia in humans (REID, MACDOUGALL, & ANDREWS, 1957). The molecular mechanism lying under the inhibition on NF-κB by those anti-inflammatory drugs such as sodium salicylate and aspirin is considered to be the prevention of degradation of IκB (Kopp & Ghosh, 1994) (Morris et al., 2009).

II Peroxisome proliferator-activated receptor-γ (PPAR-γ)

PPAR-γ is a member of the PPAR nuclear receptor superfamily. It has three forms: γ1, γ2 and γ3. The expression of PPAR-γ1 is widespread but at a low level while PPAR-γ2 and PPAR-γ3 are mainly expressed in adipose tissue (Zieleniak, Wojcik, & Wozniak, 2008).

PPAR-γ is major regulator of both glucose and lipid metabolism (Lemberger, Desvergne, & Wahli, 1996) (Spiegelman, 1998) (Kahn & Flier, 2000). Cell experiments showed that PPAR-γ regulates adipocytes differentiation by controlling the expression of numerous genes involved in differentiation and lipogenesis (Tontonoz, Hu, & Spiegelman, 1994) (Chawla, Schwarz, Dimaculangan, & Lazar, 1994). In adipocytes, such as 3T3-L1 cell line, PPAR-γ appears prior to other markers of adipocyte maturity, such as

phosphoenolpyruvate carboxy kinase (PEPCK) and adipocyte fatty acid protein 2 (aP2) (Tontonoz, Hu, & Spiegelman, 1994)(Tontonoz, Hu, Graves, Budavari, & Spiegelman, 1994). The amount of PPAR-γ in mature adipocytes is much higher than preadipocytes (Lemberger et al., 1996). Researchers found that PPAR-γ interacts with other transcription factors like CAAT/enhancer binding protein α (C/EBP α) promoting adipogenesis (Brun, Kim, Hu, & Spiegelman, 1997) (MacDougald & Lane, 1995).

Activation of PPAR-γ improves insulin sensitivity. Since stimulating PPAR-γ enhances adipogenesis, this leads to more adipocytes with smaller sizes without excessive energy intake (Spiegelman, 1998). Smaller adipocytes are usually sensitive to insulin (Hallakou et al., 1997). Because insulin stimulates glucose uptake and is antilipolytic, the adipogenesis function of PPAR-γ helps to maintain glucose homeostasis and a relatively lower level of lipolysis thus lower levels of free fatty acids (Spiegelman, 1998).

It also has important anti-inflammatory functions. PPAR-γ attenuates the development of inflammation by inhibiting NF-kB transcriptional activity in macrophages (Tilg & Moschen, 2008) and increasing the uptake and storage of free fatty acids in adipose tissue (Moller & Berger, 2003). By influencing NF-κB, proinflammatory factors such as TNF-α and monocytechemotacticprotein-1 (MCP-1) are downregulated. Previous research also shows that PPAR-γ can reverse macrophage infiltration in adipose tissue (Xu et al., 2003).

PPAR-γ activating drugs are commonly used to treat Type II diabetes. Numerous studies show that these drugs also have anti-inflammation functions. In a Type II diabetic rat

model, treatment of pioglitazone, which is PPAR-γ agonist, improved insulin sensitivity (Ko et al., 2008). The researchers also found that levels of inflammatory markers like NF-κB, MCP-1 were downregulated. Macrophage infiltration in adipose tissue was reduced dramatically as well. Thus, it is of interest to find dietary phytochemicals that possess the ability to enhance PPAR-γ that may lead dietary means to treat or prevent obesity-related morbidities.

Bioactive food components (BFCs)

Diet in general plays an essential role in the cause, development, management and prevention of diseases. On one hand, high consumption of certain food components such as sugar and saturated fat are risk factors for coronary heart disease, obesity and diabetes (Mann, 2002). On the other hand, many foods posses the ability to prevent or improve metabolic disorders (Riccardi, Capaldo, & Vaccaro, 2005). Functional foods refer to foods that have health benefits besides their nutritive value or play a role in prevention or management of diseases such as immune disorders, metabolic disorders, cardiovascular diseases and cancer (J. A. Pennington & Hubbard, 2002). The beneficial components in functional foods are referred to as bioactive food components (BFCs). BFCs include a wide range of chemical compounds such as phenolic acids, plant sterols, indoles, flavonoids, carotenoids, fatty acids, allyl and diallyl sulfides (J. A. T. Pennington, 2002). BFCs can regulate numerous metabolic processes that are beneficial to disease treatment or prevention and can be incorporated into people's diet, thus, they are excellent therapeutic tools.

Previous research has identified some BFCs that have beneficial properties. One widely used BFC is ephedrine, an extracted from Ephedra Sinica (also called Ma Huang). Animal studies showed that ephedrine reduces body weight of rats and mice mainly by increasing energy expenditure (Massoudi, Evans, & Miller, 1983). Human studies showed that ephedrine has thermogenic effects after intake in the short term (less than 24 hours) (Shannon et al., 1999), but longer term studies (more than 2 weeks) did not find a significant difference between ephedrine and placebo on an energy-restricted diet (Astrup, Breum, Toubro, Hein, & Quaade, 1992). It is thought that ephedrine acts via the central nervous system to execute its thermogenic, lipolytic and anorectic functions. Ephedrine also plays a role in delaying the gastric emptying in the study by Jonderko K et al (Jonderko & Kucio, 1991). However the safety of ephedrine is a concern. There are 140 reports reviewed by Haller CA et al documenting adverse events related to using supplements containing ephedra alkaloids (Haller & Benowitz, 2000). The possible health risks included cardiovascular symptoms such as hypertension, tachycardia, arrhythmia, myocardial infarction, cardiac arrest or even sudden death; and symptoms related to the central nervous system such as stroke, transient ischemic attack and seizure. Because of the possible adverse effects of ephedrine, ingredients containing ephedrine alkaloids are banned in the United States.

Caffeine is found in many kinds of plants such as tea, coffee, guarana and cacao beans. Caffeine working as an antagonist of adenosine, inhibits phosphodiesterase, and prolongs

the release of noradrenaline resulting in increased concentration of cyclic AMP in cells. Tulp et al. found that caffeine stimulated thermogenesis and weight loss in both lean and corpulent rats (Tulp & Buck, 1986). Short-term studies showed that caffeine consumption stimulate metabolic rate and increase thermogenesis in both healthy and obese individuals (Acheson, Zahorska-Markiewicz, Pittet, Anantharaman, & Jequier, 1980). But there are few studies testing the chronic effects of caffeine on metabolism. Some studies showed that caffeine also can increase lipolysis and fat oxidation, but results are not consistent (Bracco, Ferrarra, Arnaud, Jequier, & Schutz, 1995) (Graham, Helge, MacLean, Kiens, & Richter, 2000).

Catechin polyphenols such as epicatechin and epigallocatechin gallate exist in tea in high quantities. Catechin polyphenols inhibit catechol O-methyl-transferase, which is the enzyme responsible for degrading noradrenaline (Shixian, VanCrey, Shi, Kakuda, & Jiang, 2006). As a result, catechin polyphenols increase the levels of noradrenaline or keep noradrenaline at a high level for a longer time. The functions of tea catechins have been tested in both animal and human studies. By intraperitoneal injection of green tea polyphenols, acute effects of epigallocatechin gallate, but not other catechins, on endocrine systems of rats were investigated by Kao et al (Kao, Hiipakka, & Liao, 2000). Epigallocatechin gallate reduced body weight and blood levels of cholesterol, triacylglycerol, glucose and insulin. Some human studies of catechins also gave similar conclusions. Dulloo et al found that a green tea extract increased 24-hour energy expenditure and fat oxidation (Dulloo et al., 1999), but caffeine alone in amounts equal to those found in the green tea extract did not affect 24-hour energy expenditure. The data

from this study suggest that compounds in the green tea extract other than caffeine influence thermogenesis and fat oxidation in human. However, the study by Kovacs et al showed that green tea treatment did not help for a long-term weight control (Kovacs, Lejeune, Nijs, & Westerterp-Plantenga, 2004). Based on those previous studies, tea catechins might have some influence on thermogenesis or fat oxidation, but that could also be affected by intakes of background caffeine and some other compounds in tea extracts. Despite the effects on energy metabolism, long-term effects of catechins on body weight are unknown.

Ginsenosides are the bioactive compounds in ginseng. They are found to have anti-hyperglycemia, insulin sensitization, islet protection, anti-obesity and anti-oxidant activities (Vuksan & Sievenpiper, 2005). A study by Jin-Taek et al found that ginsenoside Rg3, which is a red ginseng rich constituent, actives AMP-activated protein kinase (AMPK) and inhibits PPAR-γ activity and adipogenesis (Hwang et al., 2009). Another study, by Ni et al, investigated the role of ginsenosides on PPAR-γ mRNA expression and metabolism of glucose and fat on type II diabetes patients (Ni, Yu, & Yang, 2010). The results showed that after 2 weeks of ginsenosides treatment, PPAR-γ expression was increased while the levels of blood cholesterol, TAG and glucose were decreased in those patients. Although those two studies had different results about the role of ginsenosides on PPAR-γ expression, both of them showed that ginsenosides have potential to improve obesity and type II diabetes.

Berberine, a quaternary ammonium salt found in rhizome coptidis, has already been used as an oral hypoglycemic agent (Yin, Xing, & Ye, 2008). It also has anti-obesity and anti-dyslipidemia functions. The possible mechanism is linked to inhibition of mitochondrial function, activation of AMPK pathway, glycolysis stimulation, induction of low-density lipoprotein receptor expression and adipogenesis suppression (Yin et al., 2008).

However, data of the previous studies concerning natural food compounds are usually not complete or are inconsistent. And most data about natural food compounds are based on epidemiological studies making it is hard to draw a cause and effect relationship. The focus of this project is to characterize BFCs which decrease TAG accumulation in cells by altering relative TAG metabolic pathways. Moreover, identifying specific those BFCs might also help for the future research to find out the etiology of obesity, type II diabetes and other metabolic diseases.

CHAPTER 2 THE ROLE OF HERBAL EXTRACTS ON FAT METABOLISM

Overview

Prevalent diseases such as obesity and type II diabetes (T2D) associate with disorders of lipid metabolism and mild to severe inflammation. Undoubtedly, diet plays a major role in disease susceptibility. Consumption of specific foods increases the risk for metabolic disorders such as obesity, T2D and coronary heart disease. On the other hand, many dietary components have preventive or therapeutic effects concerning disease incidence. The physiological benefits of these "functional foods" are beyond basic nutritional value. In this project, we aimed at identifying and characterizing bioactive food components (BFCs) through the utilization of high-through put screening of food component libraries that decrease cellular fat accumulation, thus potentially lowering susceptibility to metabolic diseases. After high throughput screening, we identified five leads that significantly reduced intracellular fat accumulation in 3T3-L1 adipocytes. These leads are from four herbs: Arctium lappa L., Dioscorea hypoglauca Palibin, Achyranthes bidentata BL. and Cynanchum paniculatum (Bge.)Kitag (two extracts are from the latter). Among the four herbs, the extracts of Arctium lappa L. and Achyranthes bidentata BL. increased the lipolysis rate in 3T3-L1 adipocytes. The expression level of hormone-sensitive lipase (HSL) in adipocytes was up-regulated by the extracts of Achyranthes bidentata BL. and Cynanchum paniculatum (Bge.)Kitag., while the same two herbal extracts down-regulated the expression level of Acetyl-CoA carboxylase α (ACC α). *De novo* lipogenesis was slightly inhibited by the leads but no statistic significance was found except the one from Cynanchum paniculatum (Bge.)Kitag. These results suggest that the five herbal extracts can decrease adiposity by affecting certain pathways in fat

metabolism which may result in lower susceptibility to metabolic diseases.

Introduction

Obesity related metabolic disorders are the center of a major health epidemic (McGinnis, 1988). In the United States, two-thirds of the population are overweight or obese and one third are obese. In 2008, 6.29% of the U.S. population were diabetic. The excessive fat accumulation, especially visceral fat, increases the risk of hyperlipidemia, insulin resistance, and other possible organ dysfunctions (Lemieux, 2001) (Schaffler, Scholmerich, & Buchler, 2005). Long term studies have showed that increasing body weight, particularly upper body fat, results in a higher incidence of T2D (Bennett, Burch, & Miller, 1971) (Knowler, Pettitt, Savage, & Bennett, 1981) (Maggio & Pi-Sunyer, 1997). Among obese individuals, most of the insulin-sensitive tissues like muscle, adipose tissue and liver tend to become insulin resistant (Braun, Zimmermann, & Kretchmer, 1996) (Oskarsson, Godwin, Gunnar, & Thomas, 1993).

As the number of patients with metabolic disorders and the cost of health care are increasing (Heffler et al., 2002), effective prevention and treatment methods become increasingly necessary. Traditional Chinese Medicine was first documented over 2,000 years ago in an ancient Chinese book *Huangdi Neijing*. Over thousands of years in development, it has been widely used in East Asia, but is considered as an alternative medical system in many western countries. However, the relatively lower cost and natural

herbs used in Traditional Chinese Medicine become more appealing and draw much attention worldwide. One of the major treatment methods is herbal medicines. It has been documented that certain BFCs derived from herbs regulate metabolic processes such as allosteric or covalent modification of proteins and gene expression (Milner, 2004). It is reasonable to assume that BFCs can modulate certain processes in metabolism. Functional foods such as certain herbs with health benefits can be incorporated into the diet, providing a great approach for widely spread health problems. Identification and characterization of novel BFCs with specific and beneficial metabolic effects are paramount for prevention and management of diseases. However, existing clinical trials and epidemiological studies show inconsistencies and do not identify cause and effect relationships. Therefore, we used the well-established 3T3-L1 adipocyte model to screen thousands of herbal extracts to expedite the identification of BFCs that can modulate adipocyte energy metabolism.

Methods and Procedures

I Materials

3T3-L1 cells were obtained from ATCC. Dulbecco's Modified Eagle Medium and trypsin were from GIBCO. Bovine calf serum was obtained from Thermo Scientific. Fetal bovine serum was from Sigma. Penicillin & streptomycin were from Mediatech.

The two herbal extract libraries ordered from HD Biosciences Co. LTD., Shanghai, China

were the anti-diabetes library (976 extracts) and anti-inflammatory library (432 extracts). A “library” here refers a group of herbal extracts thought to have similar health benefits through its use in Traditional Chinese Medicine. Herbs were extracted with one of three solvents:ethanol, water or chloroform. Subsequently, each extract underwent time-based fractionation using HPLC yielding 16 separate fractions for each herb. The collected samples were then lyophilized and stored in 96-well plates. The extracts in each well of a 96-well plate are 0.1-0.3 mg, containing approximately two to four compounds. Thus, the four libraries in total were consisted of 1,952 extracts with roughly 3,000 to 6,000 compounds from herbs or plants with putative bioactive properties.

II Dissolution of the Herb Extracts

Using the liquid handler from Beckman Coulter Inc (FX Model CAT NO 717004), we transferred 25µl DMSO into each well of the herbal extracts. After 10 minutes of vortexing, the herbal extracts/DMSO solutions were mixed three times by the liquid handler and were subsequently stored at -80°C.

III Cell Culture

The media for 3T3-L1 preadipocytes were 90% Dulbecco’s modified Eagle’s medium (DMEM) with 4.5 g/L glucose, 10% calf serum, 100 IU/ml streptomycin/penicillin and 8mg/L biotin. Two day-post confluent 3T3-L1 cells were incubated in the same media plus the addition of differentiation cocktail (10% fetal bovine serum instead of calf serum, 115 µg/ml methylisobutylxanthine, 10 µg/ml insulin and 390 ng/ml dexamethasone)

("Day 0" to " Day 3"). After 72 hours, differentiation cocktail was replaced with DMEM with 10% fetal bovine serum and 10 μg/ml insulin. The media was changed every other day.

For high throughput assays, 3T3-L1 cells were cultured in 96-well plates and each well contained 200 μl media. The media were changed every other day using the liquid handler. On "Day 0", 150 μl of the media were removed from each well and 170 μl differentiation medium were added to the cells. The cells were kept in the differentiation medium for 3 days. On "Day 3", 120 μl of the differentiation media were replaced with 160 μl DMEM containing fetal bovine serum and 10 μg/ml insulin. On "Day 5", 150 nl (~0.6-0.8 μg) of the herbal extracts in DMSO were added to the cells.

IV Oil-Red O Staining

On day 7, the cells were fixed with 3.7% formaldehyde for two minutes and rinsed with phosphate buffer solution (PBS). Then the cells were stained with 0.3% Oil-Red O working solution for 2 hours in room temperature (Oil-Red O stock solution is 0.5% Oil-Red O/isopropanol solution. Oil-Red O working solution is the stock solution and water (3:2) mixture). After rinsing with PBS until clear, 100 μl isopropanol was added to each well to dissolve the intracellular Oil-Red O dye and the plates were read by a SpectraMax M2 / M2e Microplate Reader at 510 nm wavelength. The screening and culture of cells in the 96-well format was done in conjuction with the University of Minnesota High Throughput Screening Laboratory in the Institute for Therapeutic Discovery and

Development.

V TAG Assay

Cells were harvested and homogenized by the tissue tearor and subsequently lipids were extracted using chloroform and methanol. Samples were dried down under nitrogen and re-suspended in isopropanol 1% triton x100. Intracellular TAG levels were determined using an enzymatic colorimetric kit from Stanbio Laboratory.

VI Lipolysis Assay

Culture media were harvested after cells were preincubated in serum-free phenol red-free DMEM media with herbal extracts or DMSO for 6 hours. The glycerol level was measured colorimetrically using glycerol assay kits from Sigma-Aldrich Co.

The effect of herbal extracts on lipolysis was then tested under both basal condition and stimulated condition. After two-day treatment of herbal extracts, 3T3-L1 adipocytes were starved in serum-free phenol red-free media for two hours with herbal extracts or DMSO. Then for each group, triplicate wells were treated with 1μM isoproterenol while other three wells maintained the same. Media were harvested after adding isoproterenol for an hour and the glycerol level was measured using the same kit as stated above.

VII Fatty Acid Oxidation Assay

The radioactivity of [^{14}C] oleate incorporated into acid-soluble metabolites was used as an index of fatty acid oxidation. Oleate and BSA were complexed using the same method as in the fatty acid uptake assay. On “day 7”, after 2 hours starvation in serum-free DMEM with herbal extracts or DMSO, cells were incubated in the same media as in the fatty acid uptake assay for 2 hours (100 μM oleate complexed with BSA (FA: BSA = 5) with 1 μCi/ml [^{14}C] oleate). Media was collected and perchloric acid was added to the media. After sitting overnight and centrifuge, the supernatant was used for measuring ^{14}C-labeled acid-soluble metabolites.

VIII Fatty Acid Uptake

After 2 hours starvation in serum-free DMEM with herbal extracts or DMSO, cells were incubated for 5 minutes in media containing 100 μM oleate with 1 μCi/ml [^{14}C]oleate which was complexed with BSA (FA: BSA = 5). Cells were harvested for chloroform-methanol lipid extraction. Fatty acid uptake was determined by cellular incorporated radioactivity using scintillation counting.

IX De novo Lipogenesis

Cells were preincubated in serum-free media for 2 hours before the assay. 1 μCi/ml [^{14}C] acetate was incorporated in the culture media and incubated with the cells for 1 hour. Cell lipid extraction was performed and the radioactivity incorporated into the total lipids was

quantified by scintillation counting.

X Gene Expression

The mRNA expression levels of seven genes, DGAT1, DGAT2, ACCα, FAS, GLUT4, ATGL and HSL were measured using real time PCR. Table 1 shows the sequences of primers for all gene expression assays. On “day 7”, mRNA was harvested from cells using TRIzol reagent. Complementary DNA (cDNA) was made using SuperScript VILO cDNA synthesis kit. Both TRIzol reagent and the SuperScript VILO cDNA synthesis kit were ordered from Invitrogen, USA. RPL32 was used as an internal standard. RT-PCR was done with the EPRESS SYBR GreenER Supermix Universal kit (Invitrogen, CA) using StepOnePlus Real-Time PCR System (Applied Biosystems, CA).

XI Statistical Analysis

All the data were presented as mean ± S.E.M.. Data were analyzed using one-factor ANOVA, followed by single comparisons between the control group and treatment groups using two-tailed t test. Statistic significance was defined using a p value ≤ 0.05.

Results

I High-throughput Screen

1. The Primary Screen

The primary screen was comprised of thirty 96-well plates of 3T3-L1 preadipocytes, which were differentiated to adipocytes using the method as explained above. The plates with herbal extracts were set up as in Figure 1. The first column and the last column on the plates only contained DMSO, which were used for control cells. Half of the control cells were differentiated at the same time as the treatment groups while the other half of the control cells were maintained in regular media during the whole process. Number 1 to 80 on the plate map represents treatments with individual extracts. Two days after the treatment, we performed the Oil-Red O assay to test the intracellular accumulation and compared the results of the treatment groups with control wells.

The inhibition ratios were calculated using this equation: inhibition ratio = (diff-x)/(diff-undiff). The average of the Oil-Red O values of the 8 control differentiated wells (as “diff” in the equation) on each plate and the average of 8 control undifferentiated wells (as “undiff” in the equation) where “x” represents the Oil-Red O value of each well treated with herbal extracts. Figure 2 shows the inhibition ratios of one of the thirty plates, as an example, treated with herbal extracts from Plate HDNC000395.

After the primary screen, 20 leads were identified. The inhibition ratios are shown in Figure 3. Seventeen of the leads (Plate 388 H04, F06, G06, D07, E07 & G08; Plate 389 C10, D10 & E10; Plate 391 H04 & B11; Plate 393 G02; Plate 394 E06 & G06; Plate 395 E06, E07 & H06) were inhibitors which significantly inhibited the intracellular fat accumulation and 3 of the leads (Plate 388 A02, B02 & C02) were activators which increased the fat amount inside of 3T3-L1 cells.

2. The Secondary Screen

The secondary screen tested the consistency of the effects of the twenty herbal extracts on intracellular fat accumulation of 3T3-L1 cells to avoid false positive results. A new set of plates with the twenty leads were set up as showing in Figure 4.

We tested the herbal extracts on duplicate plates and each of the plate has quadruplicate for each sample. 3T3-L1 cells were cultured and differentiated on two 96-well plates. Oil-Red O values performed as described above. Using the same equation as before, the inhibition ratios of the 20 leads are shown in Figure 5.

Based on the secondary screen results, five samples (number 16, 17, 18, 19 and 20) showed similar effects as in the primary screen. Table 2 contains information of the five herbal extracts. Further research was conducted these five herbal extracts.

II Dose Response

3T3-L1 cells were seeded in 24-well plates and differentiated as stated above and treated with the five leads using the same concentration as during high throughput screening. The five extracts reduced the amount of intracellular fat in 3T3-L1 cells as showing in Figure 6.

To investigate dose response, for each extract three dosages were tested. They are respectively half (3.57 µg/ml), double (14.28 µg/ml) of the original concentration and the original concentration (7.14 µg/ml); amounts are based on the assumption that each extract originally contained 0.2 mg. The oil red o values did not show significant differences of the effect on reducing intracellular lipid amount among three dosages (data not shown).

III TAG Assay

Since measuring absorbance of Oil Red O is an indirect measure of celluar TAG, we next pursued a more direct measure of TAG to confirm that the obtained results were true. Using the enzymatic, colorimetric TAG kit, we confirmed that the intracellular TAG amount of five treatment groups was significantly reduced compared to the control differentiated cells (Figure 7).

III Individual Herbal Extracts

Since the five extracts decreases TAG mass, we next attempted to identify the mechanisms through which they elicited these effects. To this end, each herbal extract was tested for its ability to modulate the major pathways that influence TAG metabolism: lipolysis, fatty acid oxidation, fatty acid uptake, de novo lipogenesis. We further analyzed the mRNA expression levels of several genes that play an important role in fat metabolism.

i. Herbal Extract 16 (from Arctium Lappa L.)

Upon analysis of the various pathways effecting TAG levels, we found that glycerol levels in the media were 25% higher in differentiated 3T3-L1 cells treated with extract 16 compared with differentiated cells receiving the vehicle under basal condition (Figure 8). For the stimulated lipolysis level, however, there was no significant difference between the control cells and treated cells (data not shown). The increase in lipolysis occurred without increased expression of the two major lipases, ATGL and HSL. Additionally, fatty acid oxidation and uptake, de novo lipogenesis and expression of other genes involved in adipocytes energy metabolisum were unaltered by extract 16.

ii. Herbal Extract 17 (from Dioscorea hypoglauca Palibin)

No significant changes in rates of lipolysis, fatty acid oxidation, fatty acid uptake, *de*

novo lipogenesis or the expression levels of ATGL, HSL, Glut4, ACCα, FAS, DGAT1, DGAT2 were observed in response to extract 17 (Figure 9). Thus, the mechanism through which this herb influences TAG levels remains unknown.

iii. Herbal Extract 18 (from Achyranthes bidentata BL.)

Herbal extract 18 up-regulated the basal lipolysis rate and the mRNA expression of HSL in 3T3-L1 adipocytes (Figure 10). Same as the herbal extract 16, the stimulated lipolysis level was not affected by the extract 18 significantly (data not shown) compared to that in the control adipocytes. Although de novo lipogenesis was unchanged, ACCα expression was also reduced by extract 18. No effects on fatty acid oxidation or uptake or the expression of other genes were observed.

iv. Herbal Extract 19 (from Cynanchum paniculatum (Bge.) Kitag.)

As shown in Figure 11, the expression level of HSL in adipocytes treated with herbal extract 19 was significantly higher than control cells while level of ACCα mRNA was down-regulated by this extract. The glycerol concentration in the culture media was slightly higher in cells treated with extract 19, but no statistic significance was found. Similarly, the *de novo* lipogenesis rate was inhibited but there was no statistic difference either.

v. Herbal Extract 20 (from Cynanchum paniculatum (Bge.) Kitag.)

Herbal extract 20 from Cynanchum paniculatum (Bge.) Kitag., the same herb as herbal extract 19 inhibited lipolysis rate, fatty acid oxidation and de novo lipogenesis in 3T3-L1 adipocytes markedly (Figure 12). The expression of ATGL, HSL, Glut4, ACCα, FAS, DGAT1 and DGAT2 in the cells was down-regulated after 2-day treatment of herbal extract 20 (Figure 12).

Discussion

Chinese herbs, as one of the major treatments in Traditional Chinese Medicine, have been used historically in China and other Asian countries. As Traditional Chinese Medicine's worldwide influence increases, systematic and accurate methods are required to document efficacy, safety and potential side-effects of the herbs. The herbal extracts used in this study were fractionated using high-performance liquid chromatography (HPLC), and each extract contains approximately two to four major compounds. Based on the therapeutic indications of the herbs in Traditional Chinese Medicine, the "library" concept was adopted to organize different groups of herbal extracts and do increase the likelihood of identifying potential leads. Additionally, using fractionated extracts will expedite the identification of individual BFCs compared to the use of crude extracts.

Based on numerous studies, diabetes is more prevalent in obese people (Geiss et al., 2006) (Millar & Young, 2003). It is reasonable to assume those herbal extracts in the anti-

diabetes library may have certain effects on fat metabolism. After high throughput screenings, we found the 5 herbal extracts which were from Arctium Lappa L., Dioscorea hypoglauca Palibin, Achyranthes bidentata BL., and Cynanchum paniculatum (Bge.)Kitag. inhibited intracellular fat accumulation. Both Oil Red O staining and TAG assay results showed consistently that the cells in treatment groups contained less TAG compared to the control cells.

Further studies were then conducted to elucidate the mechanisms of the inhibition effect of the 5 herbal extracts on fat accumulation in adipocytes. Possible reasons that can cause less fat accumulation are a higher rate of lipolysis, decreased free fatty acid oxidation and/or uptake, or a lower level of *de novo* lipogenesis.

I Herbal Extract 16 (from Arctium Lappa L.)

3T3-L1 adipocytes treated with herbal extract 16 from Arctium Lappa L. for 2 days released significantly more glycerol into the culture media than the control cells. This suggests that this herbal extract increased lipolytic rate of the cells. We measured the mRNA levels of ATGL and HSL which are two of the most important enzymes involved in TAG hydrolysis pathway. Interestingly, there were no significant changes in the expression levels of ATGL or HSL in cells treated with herbal extract 16. It is possible that the activity of lipolysis enzymes was higher in those adipocytes because of covalent or allosteric modifications caused by the herbal extract. Another potential reason for a

higher level of lipolysis without changing the mRNA level is that some lipid droplet protein or other protein related to lipolysis was modified after the herbal extract treatment.

Additional research has been done regarding biological functions and activities of Arctium Lappa L.. A butanol extract of Arctium Lappa L. (commonly known as burdock) roots suppressed the secretion of antigen-induced β-hexosaminidase and downregulated the expression levels of cytokine IL-4 and IL-5 in the RBL-2H3 rat mast cells which supported anti-allergic and anti-inflammatory effects of Arctium Lappa L.. (Sohn et al., 2011). Degranulation and cys-leukotrienes levels in human basophils were strongly reduced after the treatment of burdock extract (Knipping et al., 2008). So far no studies have been done regarding the effect of Arctium Lappa L. on fat metabolism of cells or animals.

II Herbal Extract 17 (from Dioscorea hypoglauca Palibin)

Herbal extract 17 from Dioscorea hypoglauca Palibin slightly decreased *de novo* lipogenesis. We also noticed a trend that the expression levels of ATGL and HSL in the treated adipocytes were higher than the control adipocytes. However, no statistic significance was shown after the analysis.

Not much research has been done about this herb on a molecular level. Some Traditional

Chinese Medicine documents state that this herb can regulate blood sugar and cholesterol levels and has antibiotic-like effects. However, those preventive or therapeutic effects are not very well proved.

III Herbal Extract 18 (from Achyranthes bidentata BL.)

The lipolytic rate in 3T3-L1 adipocytes was increased after the cells were treated with herbal extract 18 from Achyranthes bidentata BL.. This extract also increased the expression level of HSL which can be the reason for the higher lipolysis. The expression level of Acetyl-CoA carboxylase alpha (ACCα) was down-regulated by herbal extract 18. Acetyl-CoA carboxylase (ACC) catalyzes the committed step of *de novo* synthesis of fatty acids (K. H. Kim, 1997). Between the two isoforms (ACCα and ACCβ), ACCα is the isoform mainly expressed in white adipose tissue and mammary gland, playing a major role in fatty acid synthesis (Munday & Hemingway, 1999). Affecting *de novo* fatty acid synthesis can lead to lower fat production.

Previous studies found that Achyranthes bidentata BL. extracts inhibited parathyroid hormone-induced bone resorption and prevented bone mineral density loss in ovariectomized rats which makes Achyranthes bidentata a potential treatment for osteoporosis (He, Hui, Tezuka, Kadota, & Li, 2010). Achyranthes bidentata polypeptides played a neuroprotective role in a mouse model with sciatic nerve crush injury (Yuan et al., 2010). Polysaccharide from achyranthes bidentata as a dietary supplement enhanced

immune responses of piglets which were weaned at 28 days of age (Q. Chen, Liu, & He, 2009). However, no research involving effects on energy metabolism have been documented.

IV Herbal Extract 19 (from Cynanchum paniculatum (Bge.) Kitag.)

Herbal extract 19 from Cynanchum paniculatum (Bge.) Kitag. significantly increased the expression level of HSL and down-regulated the expression of ACCα. Lower amount of ACCα affects the synthesis of fatty acid which can contribute to a lower amount of fat in the adipocytes.

Paeonol in Cynanchum paniculatum (Bge.) Kitag inhibits endogenous vasoconstrictors by attenuating intracellular calcium concentration. This makes Cynanchum paniculatum (Bge.) Kitag. a potential therapeutic tool for certain cardiovascular diseases (Li, Bao, Xu, Murad, & Bian, 2010). An ethyl acetate extract of Cynanchum paniculatum (Bge.) Kitag exhibited anti-inflammatory effects in mice and rats (Choi et al., 2006). The extract also showed sedative effects in the same study which led to anti-nociceptive effects in the animals. However, there were no studies done exploring the effect of this herb on metabolic pathways.

V Herbal Extract 20 (from Cynanchum paniculatum (Bge.) Kitag.)

The results of high throughput screening showed that herbal extract 20, which was derived from the same herb as the herbal extract 19, Cynanchum paniculatum (Bge.)Kitag., decreased the intracellular fat mass in 3T3-L1 adipocytes without affecting cell viability as measured by cellular protein abundance. For the experiments attempting to identify the mechanisms for decreased TAG, we ordered additional larger quantities of the herbal extracts. The fact that the cells treated with the extract 20 from this different preparation of extracts had lower glycerol concentration in the culture media, lower fatty acid oxidation, markedly lower *de novo* lipogenesis and less mRNA of all seven genes may suggest that the second bunch of herbal extract 20 possess slight toxicity to 3T3-L1 adipocytes which affected the normal status of the cells. However, cells treated with the extract 20 still had similar protein contents and visualing appeared normal. The reasons explaining these pronounced effects are currently being investigated.

In conclusion, we have identified 5 herbal extracts from Arctium Lappa L. (herbal extract 16), Dioscorea hypoglauca Palibin (herbal extract 17), Achyranthes bidentata BL. (herbal extract 18), and Cynanchum paniculatum (Bge.)Kitag. (herbal extract 19 and 20) reduced intracellular fat accumulation in 3T3-L1 adipocytes. The extract 16 from Arctium Lappa L. and extract 18 from Achyranthes bidentata BL. exhibited the function of increasing lipolysis rate. RT-PCR results showed that the extract 18 from Achyranthes bidentata BL. and the extract 19 from Cynanchum paniculatum (Bge.)Kitag. up-regulated the expression level of HSL. The same two extracts of Achyranthes bidentata BL. and

Cynanchum paniculatum (Bge.)Kitag. down-regulated the expression of ACCα, the rate-limiting enzyme in fatty acid synthesis. Future studies are needed to further evaluate the biochemical mechanisms through which they work and the potential of the herbal extracts to reduce adiposity in animal studies. It will be important to identify the influence on inflammatory responses as well, to further validate the potential of these extracts to prevent or treat obesity and diabetes.

Tables in Chapter 2

Table 1. Primer Sequences for Real-time PCR

Gene	Forward	Reverse
ACCα	CGGACCTTTGAAGATTTTGTCAGG	GCTTTATTCTGCTGGGTGAACTCTC
ATGL	TGTGGCCTCATTCCTCCTAC	TCGTGGATGTTGGTGGAGCT
DGAT1	TGCTACGACGAGTTCTTGAG	CTCTGCCACAGCATTGAGAC
DGAT2	AGTGGCAATGCTATCATCATCGT	TCTTCTGGACCCATCGGCCCCAGGA
FAS	CCCTTGATGAAGAGGGATCA	ACTCCACAGGTGGGAACAAG
GLUT4	AACGGATAGGGAGCAGAAACCCAA	GTGCAAAGGGTGAGTGAGGCATTT
HSL	TGTCCCTGAATAGGCACTGACACA	AGGTGGGAATCTCTGCATCACTGT
RPL32	AAACTGGCGGAAACCCAGAG	GCAGCACTTCCAGCTCCTTG

Table 2. Five leads after secondary screening

	Herb Name	Latin	Library
16	NiuBangZi	Arctium lappa L.	the anti-diabetes library
17	FenBiXie	Dioscorea hypoglauca Palibin	the anti-diabetes library
18	NiuXi	Achyranthes bidentata BL.	the anti-diabetes library
19	JieJueZhu	Cynanchum paniculatum (Bge.)Kitag.	the anti-diabetes library
20	JieJueZhu	Cynanchum paniculatum (Bge.)Kitag.	the anti-diabetes library

Figures in Chapter 1

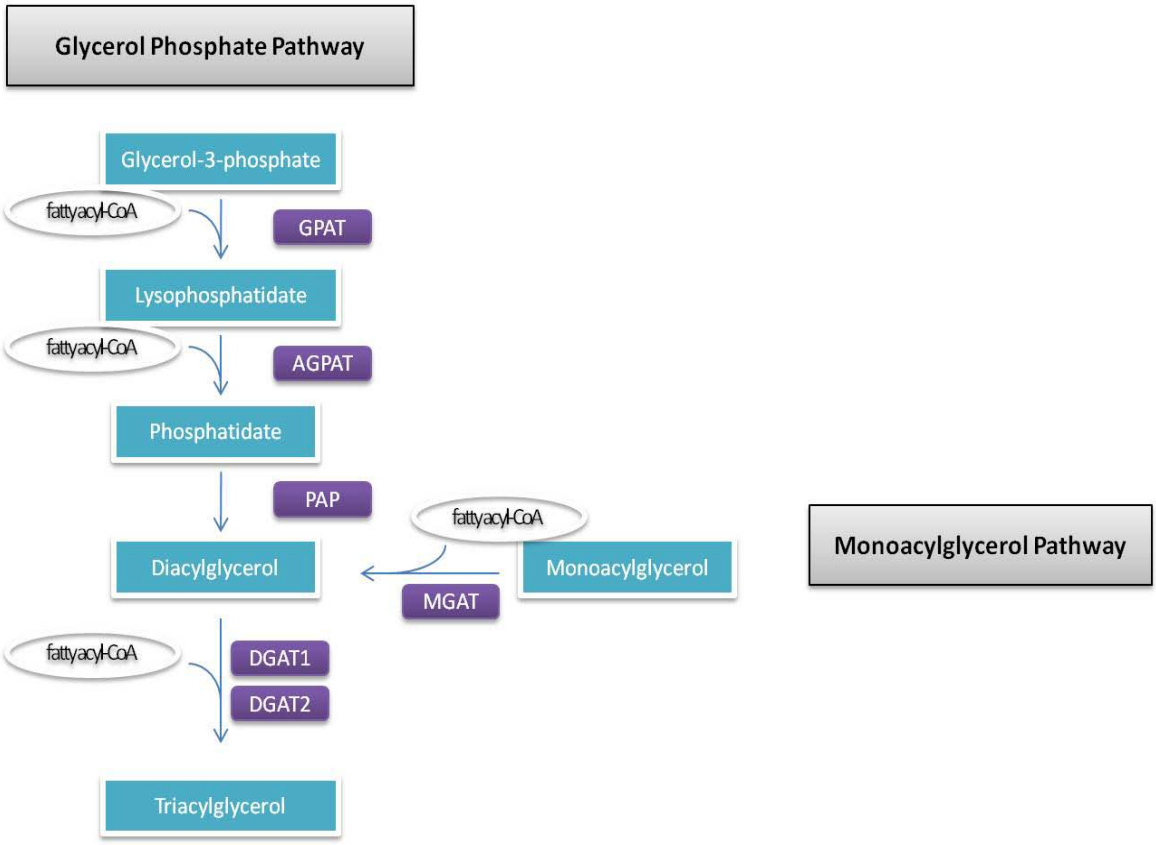

Figure 1. Two pathways for TAG synthesis: Glycerol Phosphate Pathway and Monoacylglycerol Pathway. GPAT, glycerol-phosphate (P) acyltransferase; AGPAT, acylglycerol-P acyltransferase; PAP, phosphatidic acid phosphohydrolase; DGAT, diacylglycerol acyltransferase; MGAT, monoacylglycerol acyltransferase.

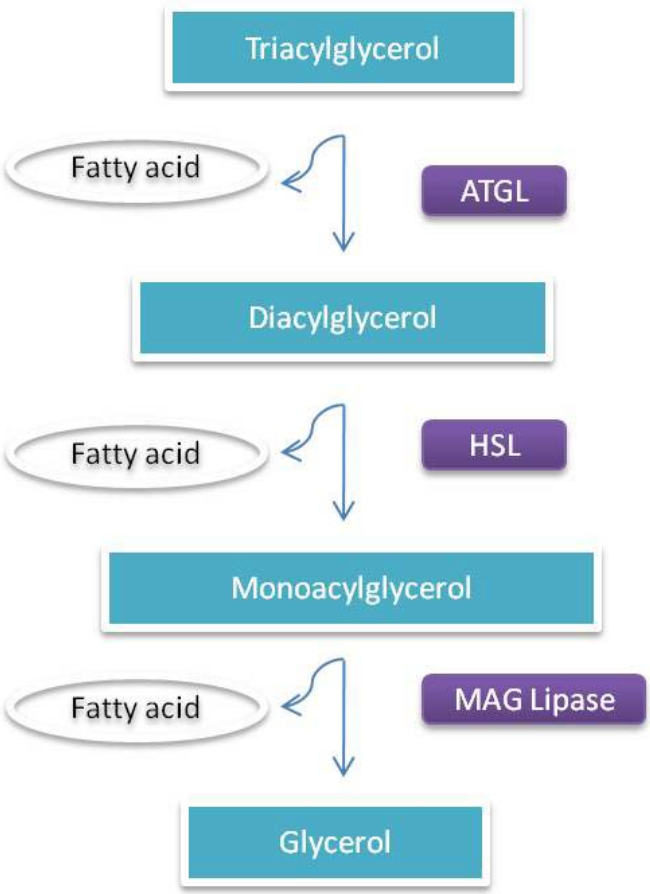

Figure 2. TAG hydrolysis. ATGL, adipose triglyceride lipase; HSL, hormone-sensitive lipase.

Figures in Chapter 2

DMSO(Diff)	1	9	17	25	33	41	49	57	65	73	DMSO(Undiff)
DMSO(Diff)	2	10	18	26	34	42	50	58	66	74	DMSO(Undiff)
DMSO(Diff)	3	11	19	27	35	43	51	59	67	75	DMSO(Undiff)
DMSO(Diff)	4	12	20	28	36	44	52	60	68	76	DMSO(Undiff)
DMSO(Undiff)	5	13	21	29	37	45	53	61	69	77	DMSO(Diff)
DMSO(Undiff)	6	14	22	30	38	46	54	62	70	78	DMSO(Diff)
DMSO(Undiff)	7	15	23	31	39	47	55	63	71	79	DMSO(Diff)
DMSO(Undiff)	8	16	24	32	40	48	56	64	72	80	DMSO(Diff)

Figure 1. Plates setup for the primary screen. Diff=for control differentiated cells; Undiff=for control undifferentiated cells. Number 1-80 are 80 herbal extracts. n=1.

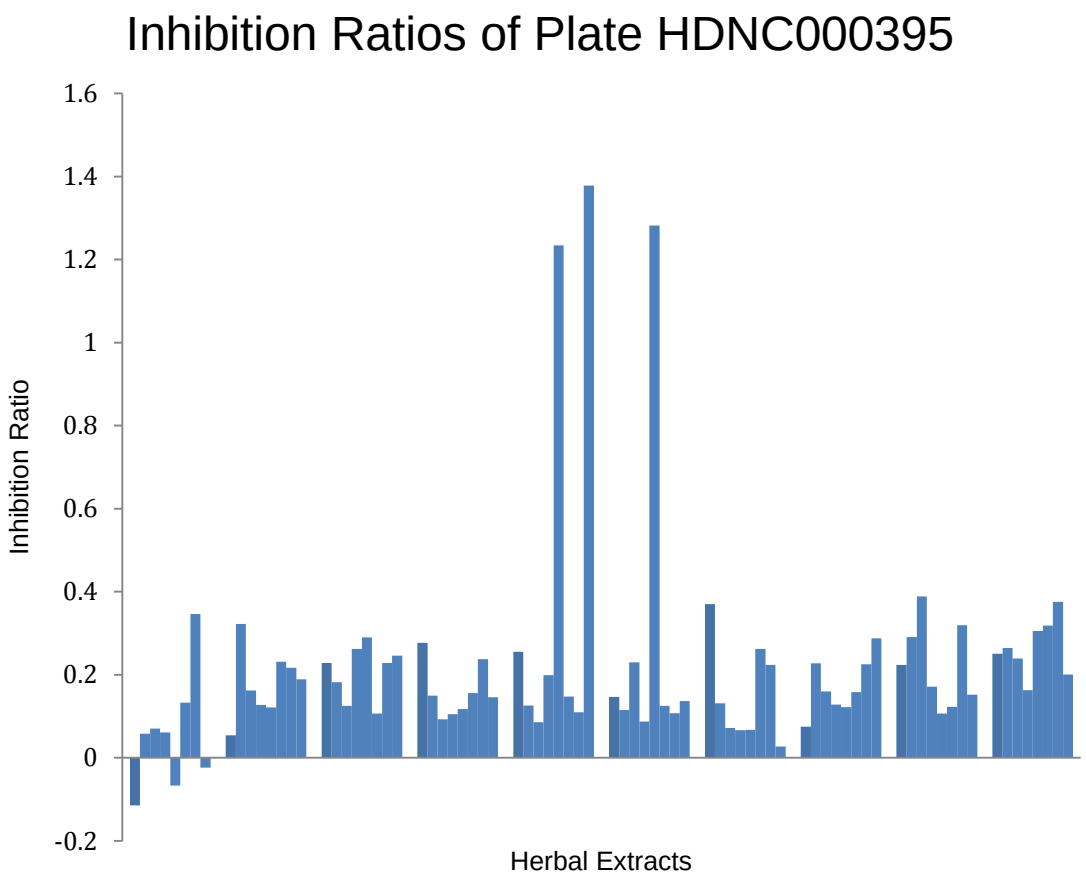

Figure 2. Inhibition ratios of herbal extracts on Plate HDNC000395. x axis represents different herbal extracts on the plate. Because the first and the last columns on the new set of plates only have DMSO, there are only 10 columns used for herbal extracts. Each series means each row on the 96-well plate.

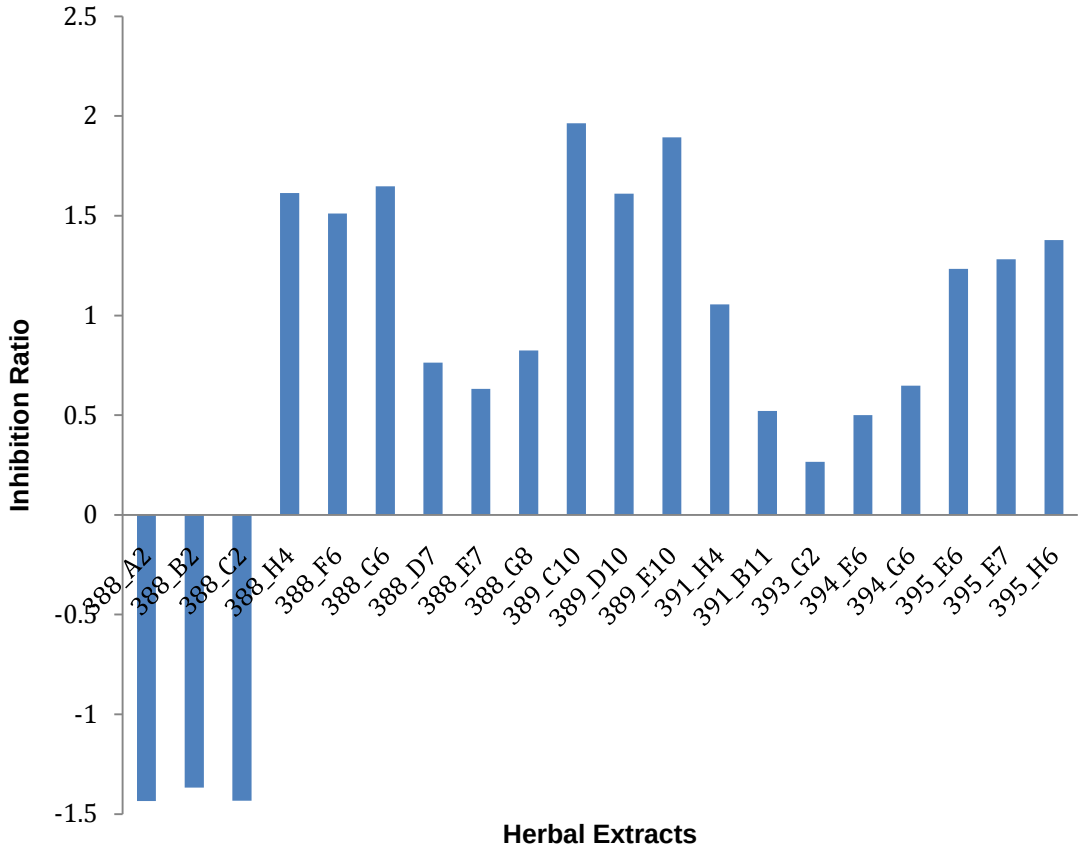

Figure 3. Inhibition ratios of 20 “leads” indentified after the primary screening. n=1.

DMSO(Diff)	1	3	5	7	9	11	13	15	17	19	DMSO(Undiff)
DMSO(Diff)	1	3	5	7	9	11	13	15	17	19	DMSO(Undiff)
DMSO(Diff)	1	3	5	7	9	11	13	15	17	19	DMSO(Undiff)
DMSO(Diff)	1	3	5	7	9	11	13	15	17	19	DMSO(Undiff)
DMSO(Undiff)	2	4	6	8	10	12	14	16	18	20	DMSO(Diff)
DMSO(Undiff)	2	4	6	8	10	12	14	16	18	20	DMSO(Diff)
DMSO(Undiff)	2	4	6	8	10	12	14	16	18	20	DMSO(Diff)
DMSO(Undiff)	2	4	6	8	10	12	14	16	18	20	DMSO(Diff)

Figure 4. Plates setup for the secondary screen. Diff=for control differentiated cells; Undiff=for control undifferentiated cells. Number 1-20 are 20 herbal extracts. Secondary screen was done on duplicate plates and each plate has quadruplicate for each herbal extracts.

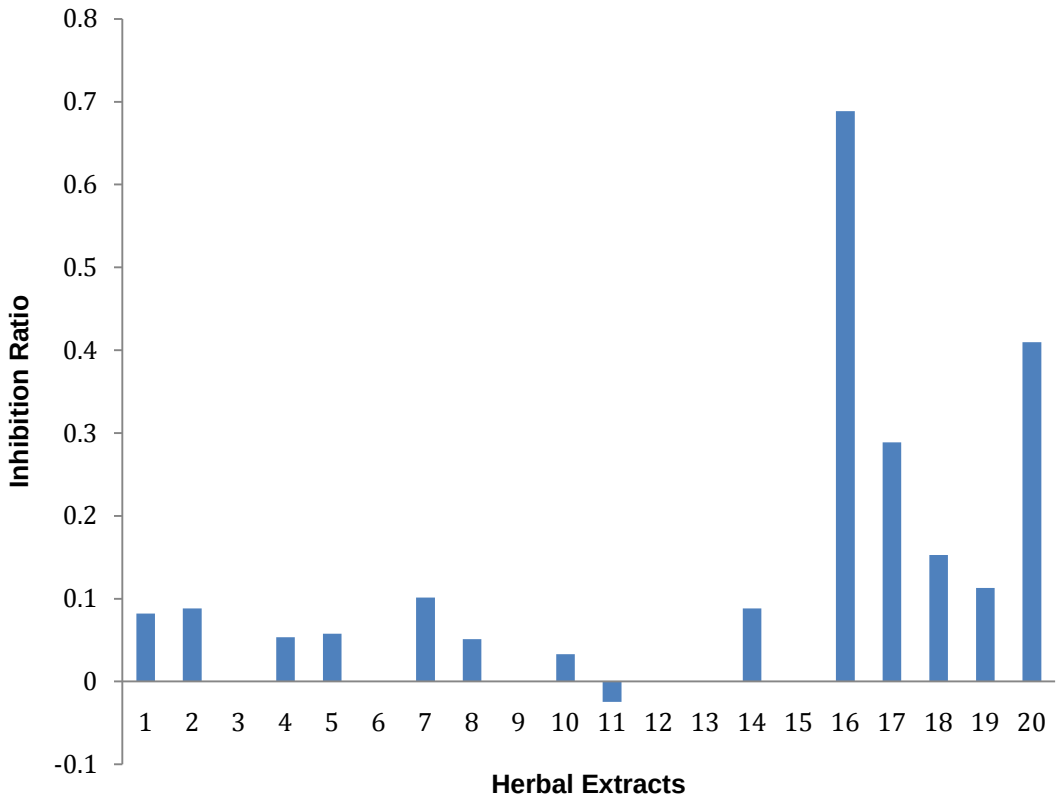

Figure 5. The inhibition ratios of 20 leads. X axis represents each herbal extract. From 1 to 20, they are Plate 388 A02, B02 & C02; Plate 388 H04, F06, G06, D07, E07 & G08; Plate 389 C10, D10 & E10; Plate 391 H04 & B11; Plate 393 G02; Plate 394 E06 & G06; Plate 395 E06, E07 & H06. Y axis represents the inhibition ratio. The experiment was done on duplicate plates. n=4.

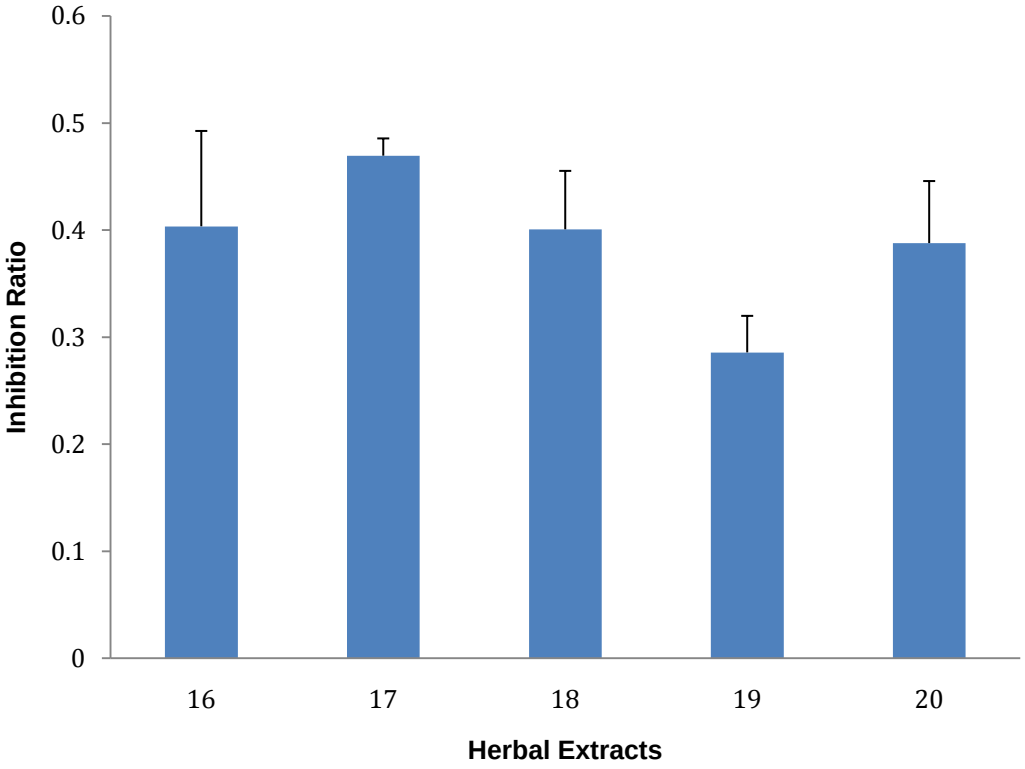

Figure 6. Inhibition ratios of five leads on 24-well plates. n=3. The data are presented as means ± S.E.M.

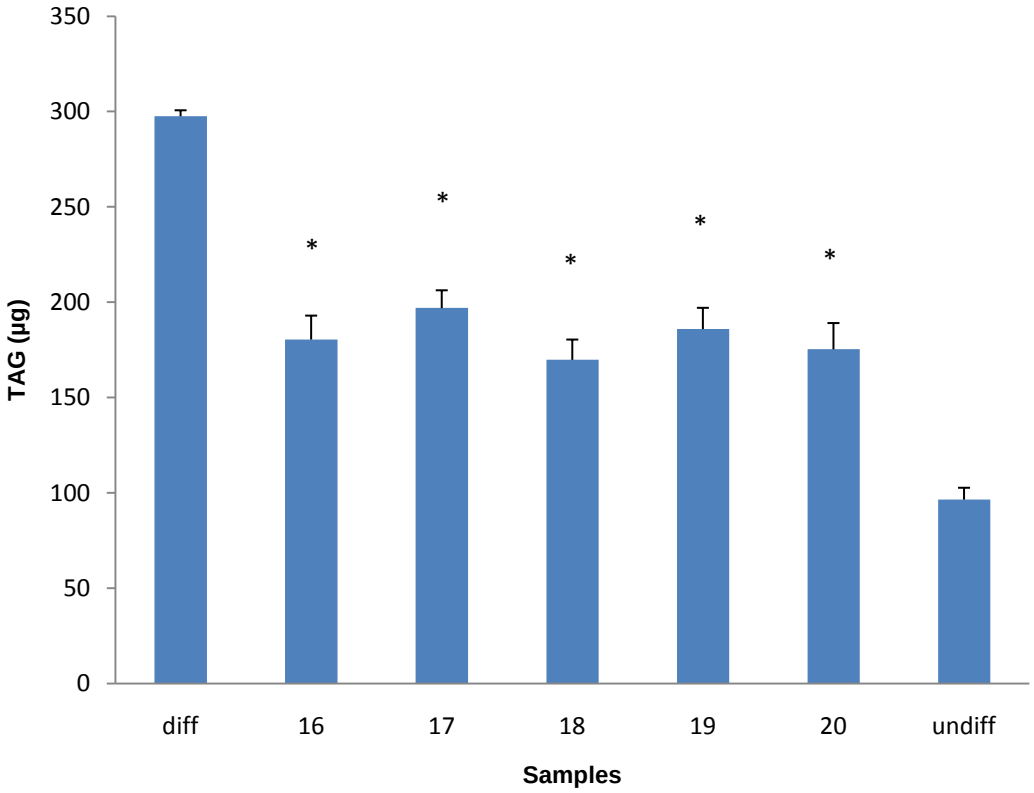

Figure 7. Intracellular TAG amount of the 5 treatment groups and control groups. This experiment was done in triplicate. The data are reported as means ± S.E.M. *$p<0.05$.

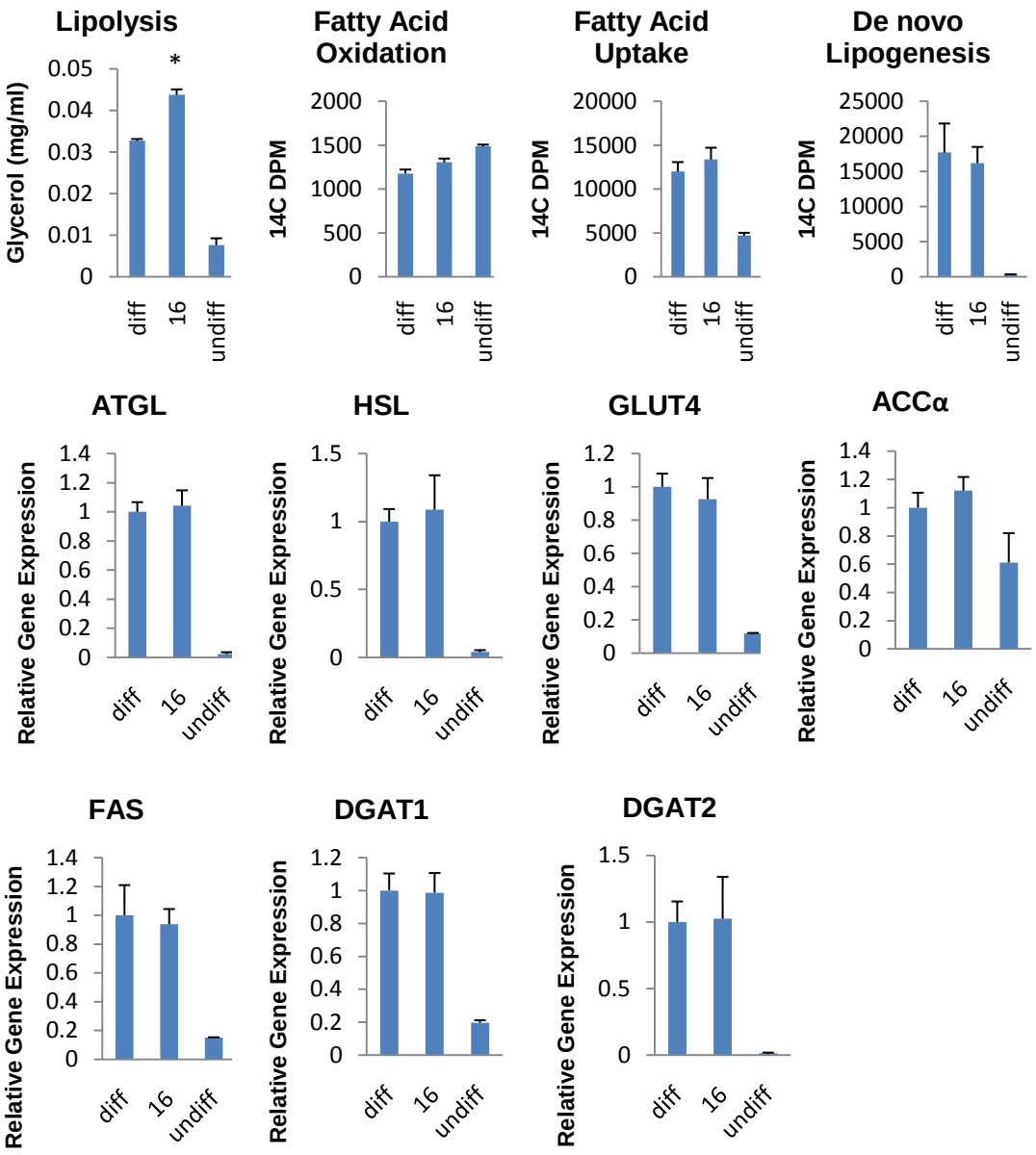

Figure 8. Results of lipolysis, fatty acid oxidation, fatty acid uptake, de novo lipogenesis assays and RT-PCR performed on cells treated with herbal extract 16. n=3. The results are reported as means ± S.E.M. *p<0.05.

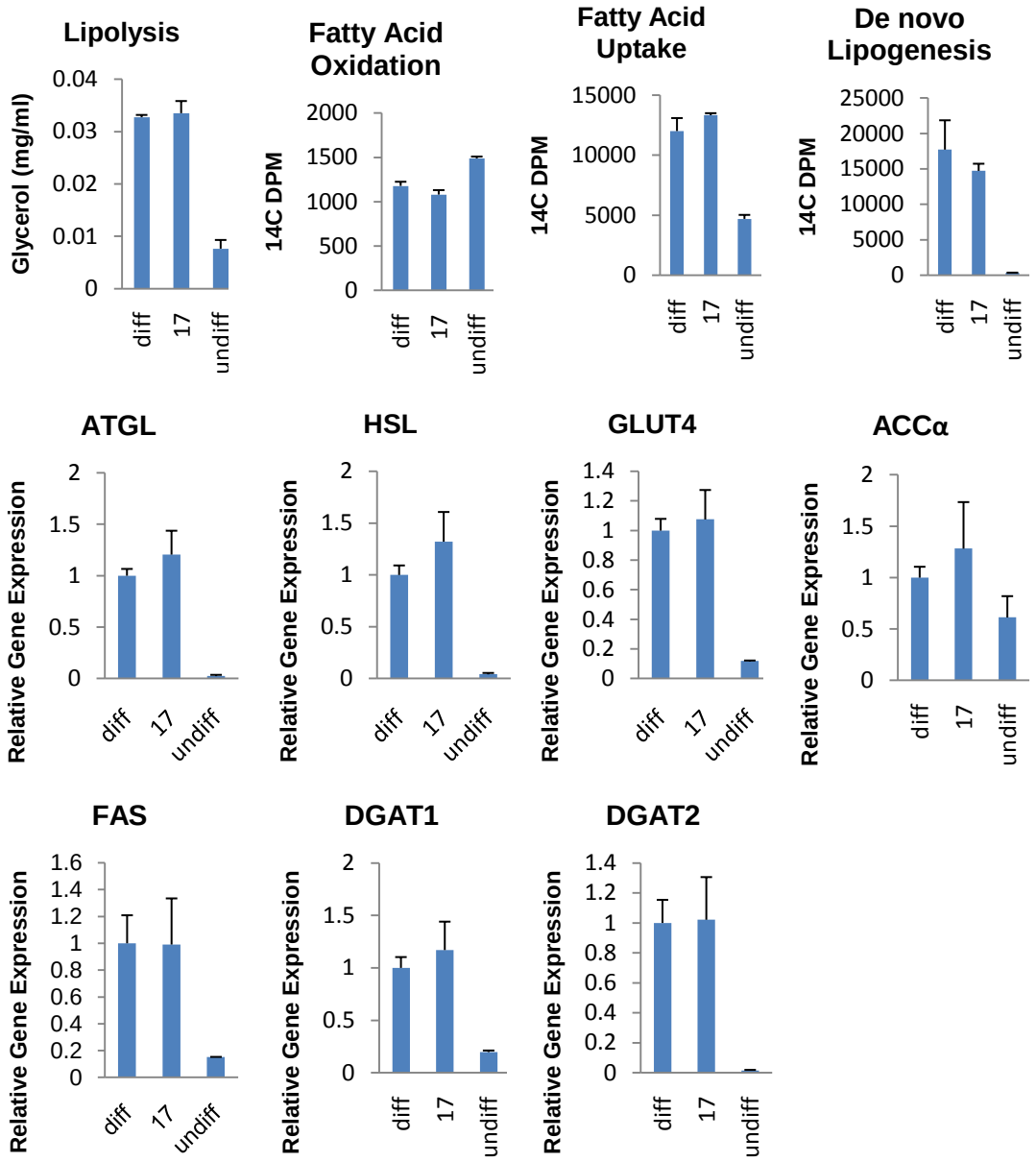

Figure 9. Results of lipolysis, fatty acid oxidation, fatty acid uptake, de novo lipogenesis assays and RT-PCR performed on cells treated with herbal extract 17. n=3. The results are reported as means ± S.E.M. *p<0.05.

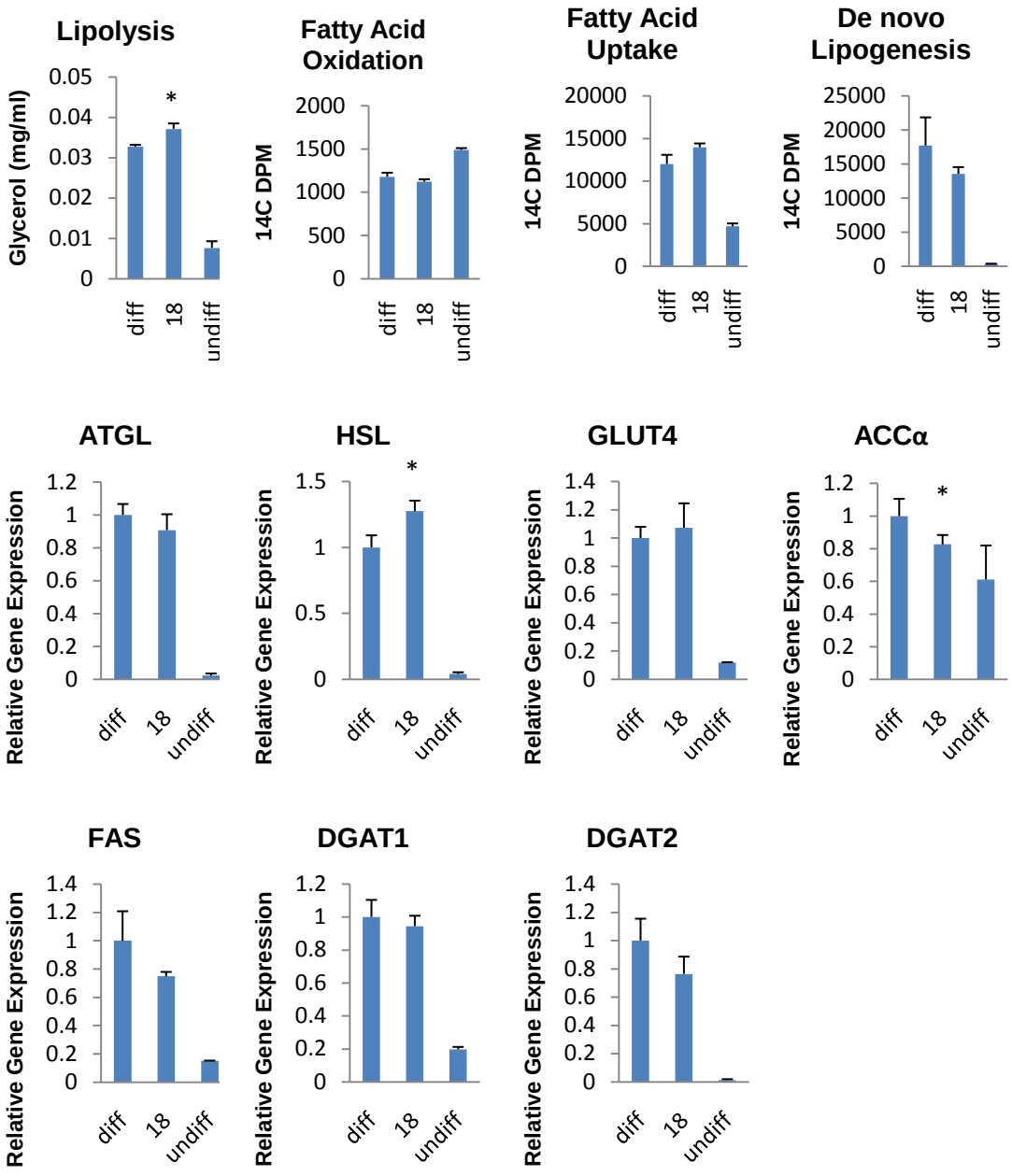

Figure 10. Results of lipolysis, fatty acid oxidation, fatty acid uptake, de novo lipogenesis assays and RT-PCR performed on cells treated with herbal extract 18. n=3. The results are reported as means ± S.E.M. *$p<0.05$.

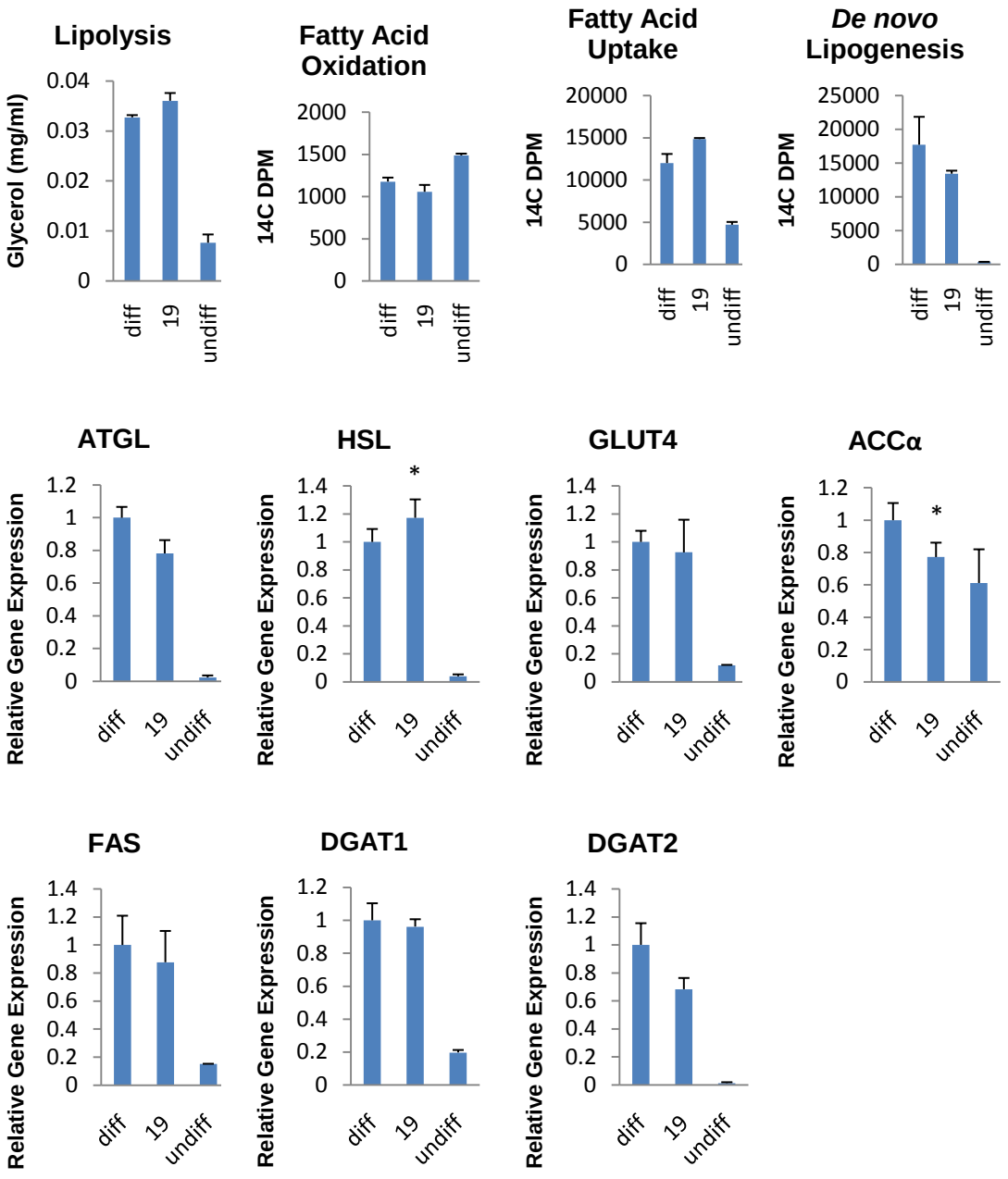

Figure 11. Results of lipolysis, fatty acid oxidation, fatty acid uptake, de novo lipogenesis assays and RT-PCR performed on cells treated with herbal extract 19. n=3. The results are reported as means ± S.E.M. *p<0.05.

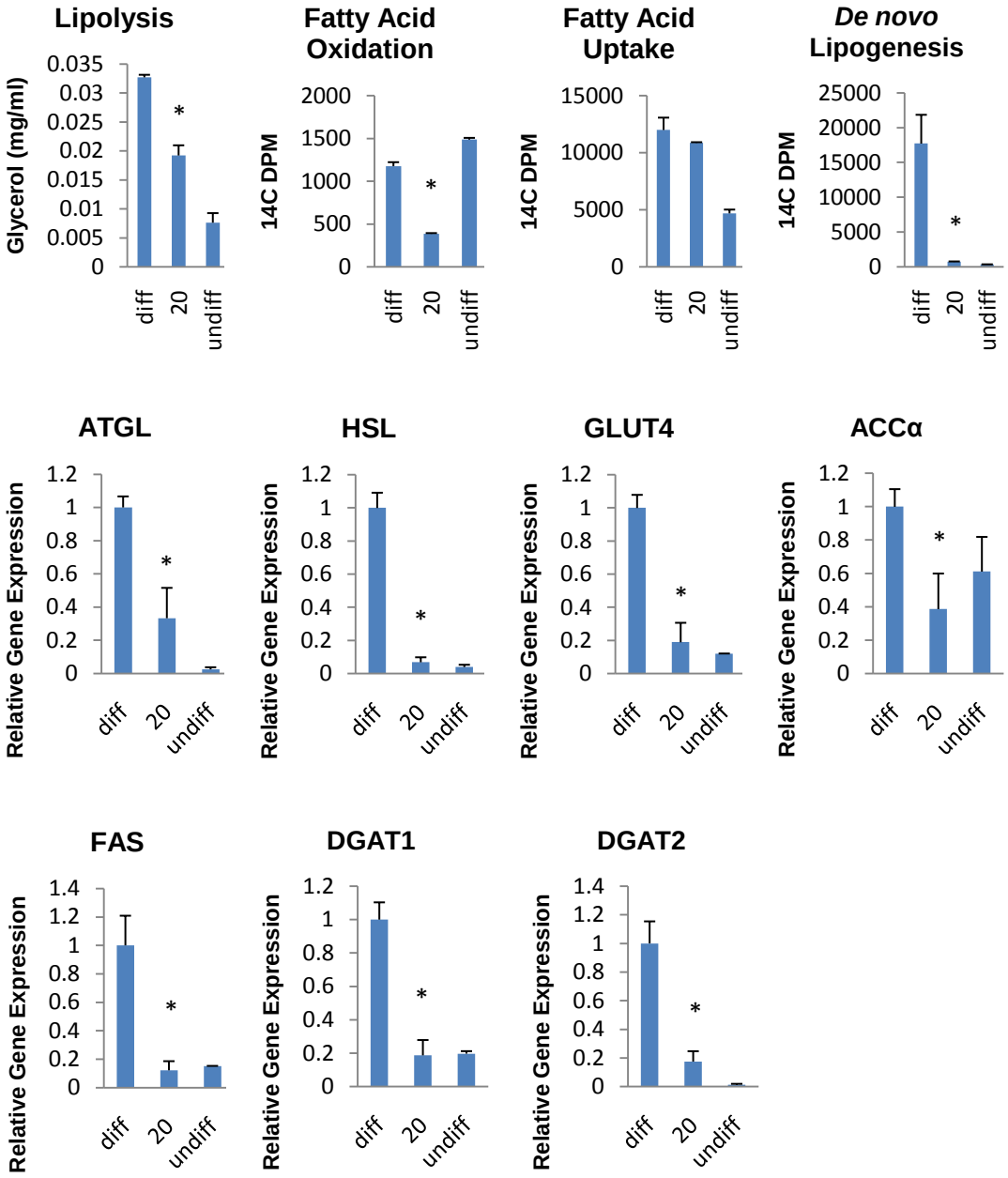

Figure 12. Results of lipolysis, fatty acid oxidation, fatty acid uptake, de novo lipogenesis assays and RT-PCR performed on cells treated with herbal extract 20. n=3. The results are reported as means ± S.E.M. *p<0.05.

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